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Sensitization To A Milk Allergen Induces Differential Behavioral And Neurological Responses In Allergic C57BL/6J And BALB/cJ Mice

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SENSITIZATION TO A MILK ALLERGEN INDUCES DIFFERENTIAL BEHAVIORAL AND NEUROLOGICAL RESPONSES IN ALLERGIC C57BL/6J AND BALB/CJ MICE

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

Table Page

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ABSTRACT

Studies have highlighted an association between food allergies and neuropsychiatric disorders such as anxiety, depression, and attention deficit hyperactivity disorder. Though the precise mechanism behind this relationship is unknown, it marks a potential novel therapeutic strategy outside of traditional neuropharmacological intervention. Food allergy is a heterogeneous disorder of the immune system characterized by an immune response that occurs reproducibly to a given food. Chronic allergen exposure in patients with mild or non-anaphylactic food allergies may cause or exacerbate psychiatric conditions. However, the variability in food allergies due to factors like genetic background introduces selection bias for individuals with overt anaphylactic reactions. To elucidate the framework of a mechanism by which peripheral non-anaphylactic food allergies cause behavioral changes, we sensitized mice to the cow's milk allergen β-lactoglobulin (BLG). This dissertation consists of three studies to characterize the behavioral effects of mild cow's milk allergy (CMA) and the underlying mechanism. The first study was to profile the behavioral symptoms of mice following sensitization to BLG and their underlying brain pathology. The second builds upon the findings of the first, to investigate changes in the brain using RNA sequencing and further histological analysis to understand what is happening in the brains of allergic mice. The third study compares two strains of mice with different genetic backgrounds and their responses to allergic sensitization. We evaluate the difference in their clinical symptoms, behavior, microbiomes, and released immune mediators in response to

allergic challenge to investigate how genetic predisposition influences the effects we observed in our model.

In study 1, male C57BL/6J mice sensitized to BLG presented increased anxiety-like behavior in the elevated-zero maze and grooming behavior tests and depression-like behavior during the tail-suspension test 24-48 hrs following allergic challenge. Since the mice sensitized to BLG were confirmed to be allergic based upon the abundance of BLG-specific IgE and IgG1, we histologically analyzed their intestines as the primary site of insult for CMA. In the ileum region of the small intestine, we observed a decrease in the tight junction protein occludin in BLG sensitized mice. A decrease in the abundance of occludin is common in intestinal inflammation and often leads to dysfunction of normal gut barrier function. Knowing male mice sensitized to BLG had a behavioral change, gut pathology, and were confirmed allergic, we then looked for evidence of brain pathology. We evaluated the status of astrocytes within the brain by staining for their structural protein glial fibrillary acidic protein (GFAP). We found that astrocytes in the substantia nigra were hypertrophic, and there was evidence of perivascular glial scarring which is common in neuroinflammatory conditions. The perivascular scarring also coincided with increased abundance of the proinflammatory cytokine, tumor necrosis factor-alpha (TNFα), which astrocytes both respond to and produce when activated. Together these data suggest CMA leads to anxiety and depression-like behavior in male mice and that the astrocyte response and signaling of cytokines like TNFα are involved in these behavioral changes.

In the second study, we investigate region transcriptional profiles of BLG sensitized C57BL/6J male mice. Across the multiple brain regions, using ingenuity

pathway analysis, we found pathways involved in inflammatory signaling, neuronal signaling, cell structural pathways, and disease states differentially activated in BLG sensitized mice. We validated some of our findings histologically using myelin basic protein (MBP) and IgG as targets based upon our previous glial findings and pathways involved in other glial cells and blood-brain barrier integrity. We observed no evidence of differential myelination through MBP in the brains of BLG sensitized mice but increased extravascular IgG in the brain parenchyma. High amounts of IgG staining within the brain implies impairment of normal blood-brain barrier function, which coincides with our previous astrocyte data.

In study 3, we compared the C57BL/6J and BALB/cJ strains because of their genetic differences in the allergy response. The differences were apparent when observing the overt anaphylactic response to BLG challenge; only the BALB/cJ mice showed significant clinical symptoms. However, both strains produced BLG-specific IgE in response to treatment, but only the BALB/cJ strain produced IgG antibodies. Despite the described differences, males of both strains demonstrated similar anxiety-like behavior though the changes were more pronounced in C57BL/6J mice. Knowing the differences in immune responses observed in these strains, we quantified the cytokines released into the plasma, finding increases in a Th2 cytokines interleukin (IL)-10, -13, and -21, in addition to various chemokines in male C57BL/6J mice, but no increases in male BALB/cJ mice. Based on the observed differences, we wanted to investigate the impact BLG-sensitization had on the microbiome. Both strains were found to have distinct profiles, and BLG-sensitization led to strain-specific changes in the microbiome. Despite vastly different microbiome profiles, when we performed brain-specific pathway

analysis of the microbiomes, the two strains had similar activation states of serotonergic, dopaminergic, addiction pathways, and various neurodegenerative diseases.

CHAPTER I

INTRODUCTION

Preface

This dissertation focuses on the systemic effects of mild cow's milk allergy (CMA). CMA and other allergic hypersensitivities are broader in classification than many realize, with some patients expressing mildly or asymptomatically. Existing evidence recognizes that populations of mild or asymptomatic patients are less likely to be formally diagnosed and exclude the allergens from their diet. In addition to the classic symptoms of allergy, CMA and other allergic diseases are found to be comorbid with various behavioral and neuropsychiatric conditions. With increasing numbers of patients resistant to neuropharmacological approaches for behavioral and neuropsychiatric disorders, increases in the diagnosis of mental disorders may be driven by these acute non-anaphylactic allergic disorders.

For this dissertation, three studies were conducted involving a non-anaphylactic CMA model and characterizing the impact of the disease on the peripheral immune system and the central nervous system (CNS). The first study examined the behavioral, gut, and brain pathology in C57BL/6J mice resulting from sensitization and challenge with a major milk allergy: β-lactoglobulin (BLG; Bos d 5), to assess the hypothesis that sensitization of mice to a milk allergen would cause changes in the CNS leading to changes in behavior. In the second study with our C57BL/6J model of CMA, we assayed

the intestinal permeability, performed brain RNAseq, and further evaluated the brain pathology to elucidate mechanisms of behavioral changes, specifically highlighting neuroinflammatory, demyelination, and blood-brain barrier integrity-based effects. The third study compared CMA-induced changes in behavior, peripheral immune factors, and gut microbiota profiles in two genetically distinct mouse strains. Each result chapter corresponding to each study provides additional introductory material to that study.

Food Allergy

Definition

The concept of allergy is ancient in the context of human history. Our knowledge of allergy goes back millennia; our oldest records date to 1000-2000 BCE China describing a "plant fever" occurring in autumn (Ring, 2014). The term "allergy" was first coined in the early 20th century by Austrian pediatrician Clemons von Pirquet circa 1906. Pirquet is quoted as stating, *"The conception that antibodies, which should protect against disease, are also responsible for disease, sounds at first absurd."* highlighting what was at the time a massive gap in our understanding of disease and immunity. Pirquet described allergy as an altered reactivity induced by what he called an "allergen," defined as a foreign substance (Silverstein, 2000). Our modern definition of food allergy, similar to Pirquet's, an adverse health effect arising from a specific immune response that occurs reproducibly on exposure to a given food (Boyce et al., 2010). Allergy is characterized as a hypersensitivity disorder; the term "hypersensitivity" describes a broad spectrum of similar conditions, in which case the typical immune tolerance to benign environmental factors is disrupted, reproducibly provoking an undesired immune

response (Boyce et al., 2010). Allergies by classical definition are type I hypersensitivity, meaning it is mediated by antibodies of the immunoglobulins (Ig) E isotype. Food allergy by this understanding is an inappropriate IgE-mediated response to specific dietary proteins.

Prevalence

The prevalence of food allergy varies wildly depending on detection methods and the studied population. Prevalence is subjected to change based on factors such as sex, age, ethnicity, and socioeconomic status; therefore, specific populations are at higher risks for developing allergy and increased severity (Rudders et al., 2014; Acker et al., 2017; Gupta et al., 2018; Willits et al., 2018). Several genetic factors involved in establishing T helper type 2 (Th2) immunity have been identified as increasing the odds of developing allergic disease, including interleukin (IL)-4, -10, -13, and specific human leukocyte antigen (HLA) haplogroups (Howell et al., 1998; Liu et al., 2004; Campos Alberto et al., 2008; Hong et al., 2009). Overall, in the United States, food allergy afflicts 3-10% of the population (Gupta et al., 2019).

Impact on Health and Quality of Life

Childhood food allergy is an incredible burden on the individual, family, and economy. Out-of-pocket medical costs for continued treatment, lifestyle changes, and emergency medical services are surface-level costs that all families typically experience but do not capture the disease's full impact. Dr. Ruchi Gupta estimated in 2013 that $5.5 billion was lost due to out-of-pocket treatment costs for services like preventative measures in the form of special diets, emergency or regular anti-allergy drugs like

antihistamines and EpiPens, and emergency room visits. The cost equates to $931/child/year in 2013 or $1051 if adjusted for inflation to the year this dissertation is published (U.S. Bureau of Labor Statistics). The addition of the costs required to maintain allergic children's safety and health is substantial and increases the economic hardship on families and can be particularly devastating in communities already struggling with poverty. The personal burdens in conjunction with economic costs lead to a total $24.7 billion cost to the U.S. economy or $27.9 billion estimated for 2021 (Gupta et al., 2013).

In addition to the direct economic burden of maintaining a lifestyle safe for a child with food allergies, other factors such as those on social and mental burdens are important. Families of children with allergies often cite the most common issues: the need for special food, increased time preparing and shopping for food, increased time preparing for school, and inhibitions on social life (Bilaver et al., 2016). A Swedish study compared case-control pairings to evaluate the different economic and intangible costs to a household with a food allergic child. Parents/guardians of children with food allergies were more likely to report feeling restricted in their career and feelings of anger, fear, anxiety, and trauma than control families. Also, parents/guardians reported children with food allergies to be restricted in their social life and more prone to feelings of anger, fear, feeling left out, and trauma, and are considered in poor health (Wai et al., 2019). These factors serve as a simplified estimation of the challenges families of children with food allergies face.

Symptoms of Food Allergy

Types and Severity

Food allergy is known to cause a spectrum of symptoms ranging in severity. The symptoms can range from the localized inflammation and disruption of the gut to systemic anaphylactic shock symptoms. Symptoms can be broadly fit into four categories: gastrointestinal, skin irritation, respiratory distress, and cardiovascular dysfunction (du Toit et al., 2010; Burks et al., 2012; Mousan and Kamat, 2016). The less severe symptoms are often considered those involving the gut and skin, whereas respiratory and cardiovascular symptoms often are signs of a life-threatening response.

In the gut, commonly identified symptoms include diarrhea, nausea, discomfort, and vomiting. Symptoms in the gut can occur in conjunction with other symptoms or, in some cases, isolation. Gastrointestinal symptoms typically can be the mildest symptoms or misdiagnosed as other gastrointestinal diseases. Skin irritation is a likely second identifying symptom, and for health care providers is the most visible condition. Allergic inflammation can manifest on the skin in the form of hives and swelling. The swelling is sometimes observed in the throat, lips, and tongue leading to difficulty swallowing or speech impairment due to the disruption of the vocal folds, larynx, and other resonant structures. The swelling is occasionally paired with paresthetic sensations around the mouth and throat.

Respiratory and cardiovascular symptoms are often the most severe symptoms. Typically, in the respiratory tract, apparent inflammation will cause wheezing, difficulty breathing, and chest tightness. These are often paired with cardiovascular symptoms,

often showing a rapid heart rate, drop in blood pressure, and dizziness. If left unchecked with emergency epinephrine injection, severe respiratory and cardiovascular symptoms will often lead to shock, loss of consciousness, and death.

In addition to the apparent clinical pathology caused by food allergies, there are often many other accompanying symptoms. For example, patients often site before symptom onset, sometimes immediately after accidental allergen exposure, feeling anxious or a lingering sense of doom or dread. Though somewhat ubiquitous, these feelings can sometimes serve as an early indicator of imminent allergic attack in patients with clear clinical history. Together these symptoms make up what is known about the spectrum of allergic anaphylaxis indicators, though often found to vary between patients in appearance and severity.

Clinical Diagnosis

The threshold for diagnosis varies depending on the test and definition used for food allergy. The Gupta paper estimates 3-10% of the percentage of the U.S. population that has food allergies. However, multiple diagnostic approaches are commonly used in cases of food allergy, potentially obfuscating results. Typical diagnostic approaches include the skin prick test (SPT) method, allergen-specific IgE positive test, self-reporting, and food challenge.

A Meta-analysis by Roberto Rona in 2007 reviewed 934 MEDLINE articles for food allergy incidence. From this dataset, it was segregated by aspects like age and method of testing for allergy. Self and maternal reports of food allergy tend to be the most liberal estimates. The patients and caregivers have the most firsthand experience.

Therefore, they are prone to over-identifying any perceived food-induced discomfort as an allergy. Thus, diagnosis based on self-reports estimates that between 3% and 35% of people are allergic. Some studies in the meta-analysis followed up self-reports with other tests like SPT or allergen-specific IgE . The use of secondary validation confirmed that 2-5% of the population is allergic.

SPT is a standard method for assessment of allergy using in clinics. The skin is punctured with a needle coated with a small amount of the potentially offensive allergen; a positive response is when the area swells. The size of the swelling of the region, also called a wheal, is typically associated with the allergic reaction severity. Rona's meta-analysis found that pure assessment via SPT found 7-17% of the population is believed to be allergic.

One of the most important biomarkers for the establishment of allergy is the production of allergen-specific IgE. Thus blood-based detection of allergen-specific IgE is another commonly used clinical diagnostic method for food allergies. Studies in the meta-analysis that used blood-based enzyme-linked immunosorbent assay (ELISA) for allergen-specific IgE found an estimated 4-6% of the population is allergic.

Finally, food challenge-based assessments, including double-blind, placebo-controlled food challenge (DBPCFC), are common ways of identifying allergic individuals. These methods are considered the "gold standard" of diagnosis but are considered the most conservative assessment. Studies estimate that 1-10.8% of people are allergic in the meta-analysis, which is the lowest bottom range limit (Rona et al., 2007). A likely reason for this phenomenon is that DBPCFC tests only identify patients with severe allergic reactions and are less likely to identify mild or non-anaphylactic patients.

As a result, these tests are prone to underestimate the portion of the population that is allergic. Therefore, under the umbrella of allergic individuals, there are subpopulations that are highly anaphylactic and more mild or subclinical responders.

Atypical or Non-anaphylactic Food Allergies

As previously introduced, allergy is a broad-spectrum disease. Allergy has both immediate symptoms in addition to late phase symptoms that arise hours after exposure. Due to variation in symptom severity, typical local effects arise from food allergies in the gut, but systemic insults are not always present. In the case of CMA specifically, ~31% of diagnosed patients have severe responses to the allergen, meaning it is more likely for patients to exhibit mild symptoms (Gupta et al., 2011). In contrast, nut allergies are more likely to induce severe responses. The variance in symptom presentation shows that some food allergies are more likely to be non-anaphylactic and, as a consequence, display differing symptoms.

Even within the framework of the classical definition of reproducible IgE-mediated immunity, there exist non-IgE mediated aspects of allergy or IgE working in concert with other factors causing specific effects. Historically more emphasis has been given to the primarily IgE-mediated immediate reactions of food allergy and less on the delayed IgG-mediated response (Hill and Hosking, 1995; Koletzko et al., 2012). The over-emphasis of the immediate or IgE response is a problem for certain patient groups; for example, it is well documented that CMA patients are more likely to demonstrate a delayed response in infancy (Dupont, 2014).

Certain symptoms are also more associated with either the IgE or IgG-mediated response. For example, we know many of the most severe classical symptoms are associated with the IgE-mediated mast cell response. However, many of the gut-associated pathologies, such as diarrhea, malabsorption, and dysmotility in addition to delayed hypotension, can be caused by a non-IgE mediated response (Dupont, 2014; Mousan and Kamat, 2016). Similarly, there are food allergy-like symptoms and, specifically, non-IgE mediated, associated it neuropsychiatric disorders such as autism. (Jyonouchi, 2008, 2009).

Patients with atypical or non-anaphylactic food allergies often display differing symptoms from those with robust IgE-mediated responses. The variance in food allergies leads to a variable response in patients depending on the allergen. CMA is one of the most likely to generate mild or delayed symptoms. The non-IgE mediated symptoms have been focused on less in a clinical setting and often are misdiagnosed. Therefore, if a patient lacks a robust anaphylactic response or present more IgG-mediated symptoms they are less likely to be diagnosed.

Development of Type I Hypersensitivity

Antigen Presentation

Professional antigen-presenting cells like dendritic cells and monocytes function as bridges between the innate and adaptive immune systems. The antigen presentation process is typically initiated in the gut either by disrupting the epithelial barrier, transferring allergens by surveillance cells like microfold cells, undigested proteins crossing the barrier, or sampling of luminal contents by dendritic cells (Menard et al.,

2010; McDole et al., 2012; Mabbott et al., 2013). In the lamina propria, a dendritic cell will uptake an antigen by phagocytosis and process it into peptides to present on an MHC II receptor. The dendritic cell then translocates to gut-associated lymphoid tissue structures like Peyer's patches or mesenteric lymph nodes to present the antigen to follicular helper (Tfh) or Th2 cells via OX40/OX40L signaling (Liu et al., 2020).

Once presented to the Tfh cell, the adaptive immune response initiates, and antibody production begins. The T cells then stimulate naïve B cells to activate antibody production. Antibody production is driven by a single gene subjected to double-stranded DNA breakage and recombination based upon secondary cytokine signals. Typically naïve B cell produces either IgM or IgD, but in the context of type I hypersensitivity, stimulation with IL-4 promotes class switching to IgE (Market and Papavasiliou, 2003). To achieve class switching, helper T cells interact with B cells via CD40/CD40L and release IL-4. IL-4, when bound to its receptor to induces Janus kinase (JAK)-signal transducer and activator of transcription (STAT) signaling, while CD40/CD40L leads to the activation of NF-κB (Ivashkiv, 1995; Warren and Berton, 1995). In the case of IL-4 signaling, JAK1/3 is stimulated and activates STAT6 via phosphorylation. With NF-κB, STAT6 binds the DNA site upstream of the ε constant locus (Cε) and acts as a transcription factor. At this point activation-induced cytidine deaminase (AID) randomly converts cytosine bases to uracil, triggering repair enzymes to cleave the uracil nucleotides and fragment the DNA upstream of the Cε region. The process of uracil conversion and DNA fragmentation also occurs at the site of the actively transcribed constant regions of Cμ, and once both regions have breaks, the two ends are ligated together by DNA repair enzymes. The ligation process excises all other constant domains

between the Cε and the VDJ sequencing, which constitutes the rest of the antibody heavy chain. Once this process is completed, the activated B cells will now produce IgE antibodies.

IgE has two aspects that make it crucial to establishing allergy as opposed to other antibody-mediated immunities. First is the cell types that possess IgE receptors. There are two types of IgE receptors, low-affinity FcεRII/CD23 and high-affinity FcεRI. CD23 receptors are found on many hematopoietic cells, such as T cells, B cells, monocytes, and eosinophils. The FcεRI, on the other hand, is expressed by basophils and mast cells which, when activated, causes degranulation of these two cell types central to the allergic response (Stone et al., 2010). The second aspect of IgE is it is notoriously unstable when unbound, having a half-life of ~2.5 days (Poulsen and Hummelshoj, 2007). However, it can be stabilized when attached to FcεRI on effector cells like mast cells, remaining for the cell lifetime (Stone et al., 2010). This process is called priming and allows for the immediate phase of the allergic response.

Lymphocyte Differentiation

A crucial defining factor of Th2 immunity is the types of lymphocytes involved and their activation. The lymphocyte classification encompasses two distinct cell families that originate from a common lymphoid stem cell. The thymus-derived T cell and the bone marrow-derived B cells arise when lymphoid cells translocate to the thymus or continue to differentiation the bone marrow, respectively (Luckheeram et al., 2012).

B cells, as previously alluded to, are the body's antibody-producing cells. During development in the bone marrow, pro-B cells restructure the genes of the heavy chain V,

D, and J regions and light chain V and J (Tonegawa, 1987). After restructuring, the pre-B cell receptor is formed and expressed by pre-B cells. The pre-B cells migrate to the spleen, where they further differentiate and become either marginal zone B cells or follicular B cells (Loder et al., 1999; Pillai et al., 2005). Once fully developed in this fashion, marginal zone B cells can become activated by an antigen, undergo antibody class switching, and form short-lived plasma cells releasing antibodies. On the other hand, follicular B cells may become either long-lived plasma cells or memory B cells (MacLennan, 1994; Liu and Arpin, 1997; Pieper et al., 2013).

When immature T cells enter the thymus, they begin as double-positive (DP) cell that express CD4 and CD8 surface markers. CD4+CD8+ DP T cells go through a process of selection to become positive for either CD4 or CD8, and then migrate from the thymus cortex to the medulla. While in the medulla, T cells fully mature and can proliferate, naïve CD4+ or CD8+ T cell then move to a secondary lymphoid organ and wait to be activated (Luckheeram et al., 2012; Hogquist et al., 2015; Kurd and Robey, 2016; Kumar et al., 2018). CD8+ T cells engage in cytotoxic action against viral infected cells, while CD4+ or helper T cells release cytokines to promote differentiation, survival, and cell death of other immune cells depending on circumstances.

CD4+ helper T cells function as the body's professional cytokine-producing cells that can further specialize into different subtypes: Th1, Th2, Tfh, and regulator T cells (Tregs). Th1 cells are intracellular pathogen combating T cells that are induced by the cytokine IL-12, IL-2, and interferon γ (IFNγ). Th1 cells, in turn, release the cytokines IFNγ, IL-2, and tumor necrosis factor α (TNFα) and promote lymphocyte proliferation. IFNγ released from Th1 cells, however, can inhibit Th2 activation (Berger, 2000;

Raphael et al., 2015). Th2 cells are important for defense against multi-cellular parasites and response stimulated by allergy. This subtype is induced by IL-4 signaling and subsequently releases IL-4, -5, -9, -10, and -13; their cytokine profile promotes the actions of monocytes, eosinophils, and mast cells while suppressing Th1 cells and promoting their subtype (Berger, 2000; Raphael et al., 2015). Tfh cells, on the other hand, are central in the activation of B cells through affinity maturation in lymphoid follicles. Differentiation is primarily controlled by a transcription factor Bcl6; in addition, Bcl6 aids in forming germinal centers. Bcl6 is believed to be induced by IL-6 and -21 (Nurieva et al., 2009; Crotty, 2014). Tfh cells reside in the T-B cell border and promote B cell survival and proliferation, as well as their somatic hypermutation. In antibody development, Tfh cells provide both the surface interaction with the B cell using CD40L and release cytokines IL-21 and IL-4, an essential signal to induce IgE production in B cells (Yusuf et al., 2010; Crotty, 2014). Lastly, Tregs are regulator T cells that suppress inflammation. Differentiation of CD4+ cells to Tregs is driven by TGF-β through the transcription factor Foxp3. The main cytokines produced by Tregs are TGF-β and IL-10; these cytokines both have suppressive effects on other T cell subtypes, mast cells, and antigen-presenting cells (APCs), further the anti-inflammatory response, and promote immune tolerance (de Waal Malefyt et al., 1991; Speiran et al., 2009; Workman et al., 2009; Raphael et al., 2015). Th cells, particularly the described subtypes, are central to the establishment and systemic effect of allergy, the balance between Th1 and Th2 activity, immunosuppression, and antibody production.

Cytokine Production

As previously alluded, Th cells are defined by their cytokine profiles. Th cells may function as the immune system's professional cytokine producer, but many other immune cells also release cytokines when activated. Notable examples include macrophages and activated mast cells (Bopst et al., 1998; Aderem and Ulevitch, 2000; Theoharides et al., 2007; Stow et al., 2009). Cytokines have a diverse range of functions depending on the cells that are receiving the signals. For the purpose of this dissertation, the function of cytokines on the Th1 vs. Th2 axis, immune suppression, and development of food allergies are discussed below.

TNF is one of the best-characterized cytokines. Soluble TNF was first identified for its ability to induce cell death and inflammation via its receptor, TNFR1. Activation of TNFR1 can lead to caspase-8 inducing apoptosis via the recruitment of TNFR1-associated death domain (TRADD) to its intracellular domain (Locksley et al., 2001) or necroptosis and inflammation via MLKL (Micheau and Tschopp, 2003; Sun et al., 2012; Kalliolias and Ivashkiv, 2016). TNFR2, in contrast, is commonly referred to as the anti-inflammatory TNF pathway. TNFR2 acts through TRAF2, leading to activation of MAPKs, NFκB, and AKT, which lead to prosurvival and repair activity (Rao et al., 1995; Probert, 2015; Kalliolias and Ivashkiv, 2016). Although TNF is one of the principal cytokines released by Th1 cells (Raphael et al., 2015), it also produced by many other cell types such as macrophages, astrocytes, microglia, eosinophils, and mast cells (Bischoff et al., 1999; Parameswaran and Patial, 2010). Mast cell-derived TNF may play a role in the late stages of allergic inflammation and/or degranulation (van Overveld et al., 1991; Bischoff et al., 1999).

IFNγ is a core product of Th1 cells. It not only promotes the differentiation of Th1 cells but also serves as a central proinflammatory signal. IFNγ acts via a JAK1/2 mechanism, phosphorylating STAT1 to induce its transcription factor activity. Macrophages are pushed to the M1 phenotype by IFNγ, which in turn are proinflammatory cells with increased phagocytic activity, antibacterial functions, and release large amounts of TNFα, IL-12, and IL-1β (Jouanguy et al., 1999; Sica and Mantovani, 2012). For T cells, IFNγ enforces the Th1 phenotype while inhibiting Th2 and Th17 differentiation by blocking IL-4/STAT6 signaling and GATA3 expression (Naka et al., 2001; Yu et al., 2004; Shachar and Karin, 2013). Another critical function of the IFNγ for Th1 cells is class switching to the IgG2a isotype in B cells (Ivashkiv, 1995). IgG2a is believed to be important for defense against bacterial infection (Kuijpers et al., 1992).

Granulocyte-macrophage colony-stimulated factor (GM-CSF), also called colony-stimulating factor 2 (CSF2), is a growth factor known to stimulate both granulocyte and monocyte production. GM-CSF is produced in endothelial cells, macrophages, fibroblasts, and activated T cells of CD4 and CD8 lineages and Th1 and Th2 cells (Griffin et al., 1990). Interestingly, resting T cells do not express GM-CSF but begin to produce the factor following stimulation with IL-3 or GM-CSF by APCs. The functional signaling between T cells and APCs suggests a role in cell function and cellular active state maintenance. GM-CSF is also induced by pro-inflammatory cytokines IL-1, IL-6, and TNFα and, when overexpressed, leads to severe inflammation due to fibrosis, macrophage expansion, and eosinophilia (Xing et al., 1996; Shi et al., 2006). GM-CSF

signals through a JAK2/STAT5 and ERK coupled multimeric receptor (Dougan et al., 2019; Hamilton, 2019).

Another Th1 factor, IL-2, is best characterized as a T cell proliferation promoting factor. IL-2 is known to promote certain classes of activated T cells, notably Th1 and Th2 cytokine release, differentiation of memory T cells, and activity of CD8+ T cells while blocking Th17 and Tfh (Ross and Cantrell, 2018). IL-2 acts through a JAK1/3 coupled receptor that activates either STAT3 or STAT5 (Ivashkiv, 1995). Evidence supports that STAT5 may be the primary transcription factor of action as knockout studies have shown a loss of both Tregs and peripheral T cell's ability to proliferate (Moriggl et al., 1999; Snow et al., 2003).

The main Th2 cytokine, IL-4, was briefly discussed earlier in the context of IgE production. IL-4 production is best characterized in Tfh and Th2 cells, but mast cells, basophils, and eosinophils are also known to release IL-4 (Xin et al., 2007). The principal function of IL-4, in addition to IgE class switching, is regulation of inflammatory cells. For example, it is known to lead macrophages to M2 activation (Stein et al., 1992), suppress Th1 differentiation (Szabo et al., 1997), and suppress TNFα expression (Hart et al., 1991). The regulation of the pro-inflammatory pathway is a result of IL-4's main antiparasitic activity. Parasites are notoriously unaffected by typical pro-inflammatory factors or involved cells, a Th2-type response being more effective at expelling parasites during certain stages of their life cycle (Moreau and Chauvin, 2010).

IL-5 is another Th2 cytokine primarily produced by Th2 cells and mast cells (Kouro and Takatsu, 2009). Similar to IL-4, IL-5 induces antibody production in B cells (Takatsu et al., 1980). IL-5 also is vital for terminal differentiation of eosinophils,

survival, and chemotaxis (Yamaguchi et al., 1988; Rothenberg and Hogan, 2006). The signaling pathway of IL-5 is propagated via JAK1/2-STAT1/5, Btk, and Ras/ERK mechanisms. The Btk signaling pathway is implicated in the B cell function and proliferation role of IL-5 signaling, while the Ras pathway seems to carry much of the eosinophil functions (Pazdrak et al., 1995; Takatsu, 1998).

IL-6 is best described as an acute and chronic phase response cytokine, often one of the first products released during tissue damage and infections (Tanaka et al., 2014). IL-6 has been linked with pro- and anti-inflammatory signaling; which includes regulating macrophage differentiation through macrophage colony-stimulating factor (M-CSF, also called colony-stimulating factor 2 (CSF2)) (Chomarat et al., 2000), dendritic maturation inhibition (Park et al., 2004), and Th1 differentiation by IL-4 signaling and IFNγ inhibition (Diehl and Rincón, 2002; Dienz and Rincon, 2009), and IL-21 induction of IgG production in B cells (Yang et al., 2016). This diverse range of functions is achieved through broad expression by various cell types, including dendritic cells, macrophages, B cells, fibroblasts, epithelial cells, and astrocytes (Kamimura et al., 2003; Dienz and Rincon, 2009). IL-6 signals through a JAK1/2/Tyk2 coupled receptor activating transcription factors STAT1/3 (Ivashkiv, 1995; Dienz and Rincon, 2009).

IL-10 is a Th2 cytokine produced by Tfh, macrophages, dendritic, and B cells in addition to the evident Th2 cells (Couper et al., 2008). IL-10's anti-inflammatory function is mostly indirect, downregulating macrophage TNFα, IL-1, and IL-12 as a means of suppressing T cell and NK cell production of IFNγ (Moore et al., 1993; Li et al., 1999a). IL-10 also functions through a JAK1-STAT1/3/5 pathway and PKB signaling (Riley et al., 1999).

IL-12 is central to the Th1/Th2 axis, promoting Th1 differentiation while suppressing Th2. IL-12 drives Th1 differentiation through IFNγ while suppressing Il-4, inhibiting Th2 (Wills-Karp, 2001; O'Shea and Paul, 2002). IL-12 is produced by macrophages, neutrophils, B cells, and dendritic cells (Heufler et al., 1996) and functions via heterodimer receptors IL-12p35 or p40. The IL-12Rs are mainly found on NK cells and T cells and act through a JAK2/TYK2 mechanism stimulating STAT3/4 phosphorylation (Desai et al., 1992; Watford et al., 2004; Ma et al., 2015; Zundler and Neurath, 2015).

IL-13 is the final of the canonical Th2 factors and is produced by Th2 cells in addition to NK T cells, mast cells, basophils, and eosinophils (Rael and Lockey, 2011). The signaling of IL-13, as previously stated, is vital in generating IgE antibodies in B cells, but also mucus secretion in goblet cells, and airway hyperresponsiveness, and fibrosis (Munitz et al., 2008). Though having their unique functions Il-4 and IL-13 have a great deal of functional overlap, IL-13, as a result, can bind to IL-4R, initiating its STAT6 pathway (Bao and Reinhardt, 2015).

Food-Allergy-Association Neuropsychiatric Disorders

History of Clinical Observations

Food allergies are often found comorbid with various neuropsychiatric and behavioral disorders. This connection has been established with clinical observations and case studies described as far back as 1916, meaning this link has been around nearly as long as our current understanding of allergies in general. A report by Hoobler in 1916 is the oldest record, which described children with allergy vaguely as "restless, fretful, and

sleepless" (Hoobler, 1916). In 1949, Davison described in detail a series of cases observed in his practice, "For a long time, it has been noted that symptoms of bizarre and unusual cerebral disturbances occur in allergic patients." Davison collected information on 428 patients with psychiatric conditions and allergies as potential "cerebral allergy" cases (Davison, 1949). A large number of the cerebral allergy patients had described themselves as "mean, sulky, irritable, unable to be pleased, crying without cause, worried, suicidal, jumpy, and indecisive," which now can easily be understood as affective disorders. More modern studies have also reported comorbidity of allergy with depression, anxiety, attention deficit hyperactivity, oppositional defiant, and autism spectrum disorders (Tryphonas and Trites, 1979; Patten and Williams, 2007; Garg and Silverberg, 2014; Shanahan et al., 2014; Ferro et al., 2016; Theoharides et al., 2016; Topal et al., 2016). The spectrum of conditions has clear overlap with the symptoms identified by Davison. In these studies, it was established that a connection exists but not whether food allergies cause or exacerbate underlying psychiatric conditions.

Comorbidity of Cow's Milk Allergy and Behavioral Disorders

Of the foods common for hypersensitivities, CMA is the second most common allergy, surpassed only by peanut allergy, afflicting approximately 20% of allergic individuals (Warren et al., 2013). Cow's milk proteins are classified into two groups: caseins and whey proteins. The major whey proteins are α-lactalbumin and β-lactoglobulin (BLG), the latter being the most common allergen in milk. BLG is absent in human milk and is highly allergenic, perhaps due to its resistance to proteolysis (Schmidt et al., 1995; Sélo et al., 1999; Iametti et al., 2002; Malacarne et al., 2002; Bossios et al.,

2011). BLG often remains in whole or partially digested peptides in the intestines, likely to stimulate an immune response when encountering immune cells (Schmidt et al., 1995).

Among the general allergic population, CMA individuals are more likely to be diagnosed with neuropsychiatric disorders. Children with milk allergy are at an increased risk of being diagnosed with attachment, oppositional defiant, attention deficit hyperactivity disorders (Shanahan et al., 2014; Ferro et al., 2016; Topal et al., 2016). Experimental mouse models have further validated these findings by sensitizing mice to milk proteins and examining their behavioral abnormalities (de Theije et al., 2014; Germundson et al., 2018). These clinical and pre-clinical observations support the role of CMA in behavior and mood disorders. However, it is necessary to investigate further the effect of CMA with a more robust behavioral study paradigm to identify a specific mechanism between the immune system and behavior.

The Role of Diet and the Immune System in Brain Function and Behavior

There is an ample amount of evidence for the immune system's regulation of the brain and behavior. As often referred to as "sickness behavior," an individual's social, physical, and eating behavior are known to be affected by infections (Miller, 1964; Hart, 1988; Yirmiya et al., 1999; Dantzer et al., 2008). Sickness behavior is likely an evolutionarily conserved behavior mechanism that forces the sick individual to focus on specific needs or change actions to better recover from the illness. The commonly identified behaviors in this condition are appetite loss, depression, and fatigue, primarily modulated by the hypothalamus-pituitary-adrenal axis (Dantzer et al., 2008). Behavioral changes are a consequence of the body's normal response to infection.

Potential mechanisms that allow the immune system to influence behavior include increased brain cytokine levels (Li et al., 2009), glial cell activation (Pardo et al., 2005; Vargas et al., 2005), and changes in amino acid and neurotransmitter concentrations. Experimental models and meta-analysis of depression have found comorbidity with inflammatory factors of both cytokines and cell-mediated activation (Maes, 2011; Liu et al., 2012). In general, immune conditions seem to be closely tied to the diagnosis of behavioral disorders such as anxiety and depression. Inflammation has been additionally implicated in various other behavioral conditions. Immune mediators and the likelihood for developing inflammatory disorders have been found in autism spectrum disorder patients (Vargas et al., 2005; Zimmerman et al., 2005; Li et al., 2009).

In addition to the direct disruption by the immune system, the other aspect of food allergy is the specific dietary element acting as the source of the sensitivity impacting the gut and behavior. It has often been reported that individuals with neuropsychiatric disorders have behavioral symptoms exacerbated by certain foods (Crayton, 1986). Compounds in food and dietary habits have routinely been linked with behavioral and emotional disorders (Khalid et al., 2016). There is also a case to be made about the impact diet and nutrition have on behavior, studies have shown that typical "western" diets showed a greater risk of psychiatric incidence (Akbaraly et al., 2009; Jacka et al., 2010; Sánchez-Villegas et al., 2011; Ruusunen et al., 2014). These assessments compound with existing knowledge on inflammation's role in behavior and mood, as diets high in fat and carbohydrates are pro-inflammatory (Smith, 1991; Liu et al., 2002; Chrysohoou et al., 2004). Western diets typically lack in critical nutrients found in abundance in eastern, modern, or traditional diets. Deficiencies in compounds such as

omega-3 fatty acids, vitamin B12, and minerals such as zinc have been linked with increased risk of depression in some cohort studies (Maes et al., 1994; Penninx et al., 2000; Murakami et al., 2010; Oddy et al., 2011). The interconnectedness of diet, gut health, inflammation, and behavior paired with the increasing diagnosis rate of behavioral disorders, including those resistant to conventional therapies, suggests that a subset of psychiatric patients may have an underlying food allergy resulting in resistance to behavior modulatory drug therapies.

Food Allergy as an Etiology and Therapeutic Target

Growing evidence supports the causative role of food allergies in neuropsychiatric disorders. With an extensive history of clinicians finding comorbidity between food allergies and neuropsychiatric diagnosis and the known role of inflammation and diet causing behavioral abnormality, discovering an immune-to-CNS signaling mechanism warrants investigation. Knowing that food allergies are highly variable and subjected to multiple factors, there is likely a subpopulation of undiagnosed allergic individuals at risk of developing behavioral disorders. With this understanding, treatment with conventional neuropharmacological therapies would be ineffective. Exploring a mechanism by which peripheral food allergies, even in mild cases, can influence behavior is invaluable and provides a novel mechanism for treating and preventing neuropsychiatric disorders. To achieve this aim, aspects of the gut-brain axis (GBA) such as absorption of neurotransmitter precursors, the microbiome, and modes of peripheral to central signaling are likely therapeutic targets.

Mechanisms of Gut-Brain Communication

An Overview of the Gut-Brain Axis

The GBA is an increasingly investigated mode of communication within the body. The GBA encompasses a broad range of factors like diet, the microbiome, and gut inflammation which influence other organ systems. This GBA's core is the bidirectional communication between the central nervous system and enteric nervous system controlling processes like digestion and satiety. The GBA consists of the collection of neural, immune, and endocrine signaling pathways between the central, autonomic, and enteric nervous systems (Carabotti et al., 2015).

Within the gut, precursors needed for nervous system function and other essential compounds not natively produced in our bodies, including those produced by bacteria, are absorbed and processed—the clearest example of this is serotonin/5-hydroxytryptamine. Our gut contains 90% of the serotonin in the body, which is derived from tryptophan. Tryptophan is an essential amino acid, which our bodies cannot produce. Tryptophan is instead absorbed from our diet with the aid of our commensal microbiota. Once absorbed, tryptophan can be transported to the CNS and converted into serotonin by the enzyme tryptophan hydroxylase (Lillesaar, 2011; Hoglund et al., 2019). Under normal circumstances, this route is sufficient to supply the necessary precursors for the nervous system; however, alteration of the microbiota via sustained changes in diet, infection, or inflammatory diseases of the gut can disrupt normal function (Holtmann et al., 2017). Subsequently, dysfunction of gut tryptophan absorption and

serotonin synthesis reduction can lead to depression and various neuro-signaling disorders.

Conversely, efferent signals from the brain to the gut are necessary for homeostatic function. Inverse to the system we have previously described, stress disrupts gut microbiota function. Experimental models have demonstrated that social stressors impact the microbiota (Galley et al., 2014; Carabotti et al., 2015). One possible mechanism of microbiota changes is the autonomic nervous system's control of secretory function within the gut, which can directly influence microbe populations in the intestine.

The Role of Gut Microbiota in the Immune and Nervous System

The microbiota has vast implications across many systems. Allergic inflammation is both a cause and effect of dysbiosis (Stefka et al., 2014; Hussain et al., 2019; Matsui et al., 2019). Food allergy as an inflammatory condition is capable of breaking down normal gut barrier function leading to changes in normal gut bacteria. Alternatively, gut bacteria act as regulators of the local immune system and, if dysregulated, make the host susceptible to food allergy. Both functions are potential mechanisms, and in addition, gut bacteria changes are commonly found in individuals with neuropsychiatric disorders, especially those paired with gut dysfunction. (Wang et al., 2011; Bunyavanich et al., 2016; Cenit et al., 2017; Pulikkan et al., 2018; Savage et al., 2018). Both the loss of neuroactive compounds produced by gut bacteria and dysregulations of the local immune system can influence behavior and brain function.

Cytokines and Their Role in CNS Communication

Cytokines are crucial to the mechanism of the gut-to-CNS signaling. As previously stated, cytokines are the primary way immune cells communicate with each other and non-immune cells. Many factors such as TNFα, IL-6, IFNγ, IL-4, and IL-10 have well-characterized interactions with the CNS, including their roles in behavior modulation (Kronfol and Remick, 2000; Zorrilla et al., 2001; Anisman and Merali, 2003; Arosio et al., 2004; Shapshak et al., 2004; Theoharides et al., 2004; Li et al., 2009; Abbott et al., 2015). Though the exact mechanisms are not entirely characterized, glial cells such as astrocytes, oligodendrocytes, and microglia, are promising as they typically act as mediators between the CNS and immune cells (Eddleston and Mucke, 1993; John et al., 2003). There is currently evidence suggesting that allergy-induced inflammation releases cytokines that relay signals directly to the CNS via afferent sensory neurons or by stimulating resident immune cells in the brain like T cells and mast cells.

Dissertation Research Objective

This dissertation aims to further our laboratory's objective to elucidate the mechanism(s) by which CMA can impact brain function and behavior. Our laboratory's focus is the communication between the peripheral immune system and the CNS leading to behavioral abnormality. Our attention has focused on exploring the relationship between BLG allergy symptoms and T cell immune predisposition, glial cell function, blood-brain barrier integrity, and host-microbiome factors. The objective of this dissertation is to explore a mechanism of BLG-allergy, which leads to behavioral changes

through a cytokine-mediated peripheral to central signaling to glial cells. This dissertation is organized into three studies aimed at addressing the following questions:

1. What is the behavioral and histopathological impact of BLG sensitization in a non-anaphylactic mouse model of CMA?

2. Which brain signaling pathways and CNS mediators are differentially activated following BLG sensitization?

3 Does genetic predisposition for a helper T cell bias influence the physiological and behavioral effects of CMA?

CHAPTER II

METHODS

Animals

Three-week-old male and female C57BL/6J and BALB/cJ mice were purchased from Jackson Laboratories (Bar Harbor, ME, U.S.A.). On arrival, mice were caged by both sex and strain, and randomly divided into groups before housed under a 12-hr light/12-hr dark cycle in a specific-pathogen-free environment at the University of North Dakota animal facility. Mice were given *ad libitum* access to whey-free rodent diet (Teklad 2018, Envigo, Indianapolis, IN, U.S.A.) and ultra-filtered water. Body weights of mice were recorded weekly to assess health and growth throughout the experiment. All procedures involving mice were approved by the University of North Dakota Institutional Animal Care and Use Committee prior to experiments.

Animal Use

Sensitization

Mice were randomly assigned to either sham or BLG-sensitization groups and given weekly intragastric oral gavage for 5 weeks (Figure 1). Sham and BLG treatment groups received vehicle solution with or without BLG. Vehicle solution contained 10 μg cholera toxin (CT; #100B, List Labs, Campbell, CA, U.S.A.) as an adjuvant in 200 μL sodium carbonate/bicarbonate buffer (0.2 M, pH 9.0), while the BLG solution contained

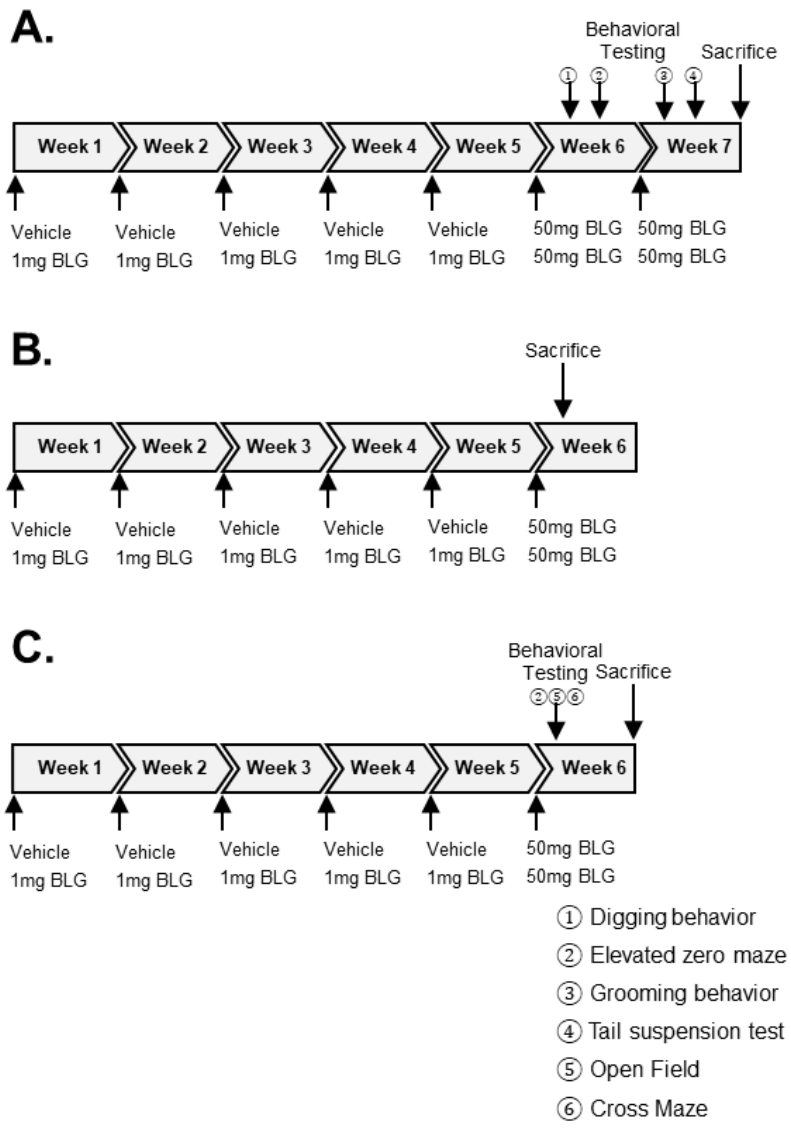


Figure 1. Schematics of the experimental timelines in the studies. Starting at 4-weeks of age, mice were given a weekly oral administration of 200 µL vehicle (carbonate/bicarbonate buffer containing 10 µg CT, pH 9.0) with or without 1 mg BLG for 5 weeks. (**A**) In study 1, during the 6th and 7th weeks, all mice were challenged with 50 mg BLG in carbonate/bicarbonate buffer (without CT), and their behavior was subsequently tested at 1- and 2-day post-challenge. One day after the last behavior test in Week 7, mice were sacrificed, and blood and tissue samples were harvested. (**B**) In study 2, During week 6, all mice were challenged with 50 mg BLG in sodium carbonate/bicarbonate buffer (without CT) and were sacrificed 1-day post-challenge. (**C**) in study 3, During week 6, all mice were challenged with 50 mg BLG in sodium carbonate/bicarbonate buffer (without CT), and their behavior was tested 1-day post-challenge.

an addition of 1 mg BLG (#L0130, MilliporeSigma, St. Louis, MO, U.S.A.) in the vehicle. Two hours prior to and following treatment, normal whey-free diet was removed, and mice were fasted.

Allergen Challenge

Beginning on week 6 (1-2 days prior to behavioral testing), mice were challenged with BLG solution to assess for anaphylactic reaction. BLG solution contained 50 mg BLG in 200 μL sodium carbonate/bicarbonate buffer (0.2 M, pH 9.0) without the presence of adjuvant. Mice were fasted for 2 hrs before gavage and observed for 30 min following gavage to assess physical responses.

Acute Physical Responses

Following challenge, physical responses were assessed. Anaphylaxis symptoms were graded on a 0-5 point scale established in Li, et al. (1999)(Li et al., 1999b). Briefly, “0” on this scale indicates no reaction, “1” notes the presence of body scratching due to skin irritation, “2” indicates swelling, redness, and reduced activity, “3” denotes respiratory distress observed by labored breathing and cyanosis, “4” indicates shock symptoms by convulsion and lack of responsiveness, and a score of “5” is when death occurs following challenge (Table 1). Secondly, we assess allergy-induced hypothermia using a MicroTherma 2T Handheld Thermometer with a RET-3 probe (Braintree Scientific, Inc., Braintree, MA, U.S.A.). Mice were returned to their home cages and allowed to rest for one day until behavior tests.

Table 1. Anaphylaxis scoring scales. Scores and symptom descriptions adapted from Li et al., 1999. (Li et al., 1999b)

0	No reaction/clinical symptoms
1	Scratching and rubbing around the nose and head
2	Puffiness around the eyes and mouth, pilar erecti, reduced activity
3	Wheezing, labored respiration and cyanosis around the mouth and tail
4	No activity after prodding or tremor and convulsion
5	Death

Behavioral Testing

Innate digging behavior was assessed by placing each mouse in an enclosure (24.8-cm width × 38.7-cm depth × 29.2-cm height) containing 5-cm thickness of clean corncob bedding. Mice were allowed to explore the enclosure freely for 20 min. The first 5 min were used to acclimate mice to the enclosure, and the following 15 min were recorded for analysis of digging behavior. The frequency of digging activity was counted from the videos by two observers blinded to the mice's experimental conditions (Figure 2A).

Mice were individually placed in empty cages, and their grooming behavior was video-recorded for 10 min after 5 min of acclimation. As with the scoring of digging behavior, two blinded observers from the video recordings counted the grooming behavior frequency. The presence or absence of grooming activity was monitored by

giving 1 or 0 points, respectively, during each of 60 × 10-sec intervals. The sum of the points for the testing period (10 min) was considered the mouse's grooming frequency (Figure 2B).

Tail suspension test (TST) was performed based on a previously described protocol. Briefly, mice were suspended by their tail from a horizontal bar with a piece of laboratory tape so that their nose was ~ 30 cm above the base of the bar support. The C57BL/6 strain is known to climb their tail; a plastic tubing piece was used to maintain the mice in the suspended position. Their attempts to escape from the position were during the 6 min video-recorded. The frequency and length of immobility and the latency to the first immobile episode were compared between groups as indications of depression-like behavior (Figure 2C).

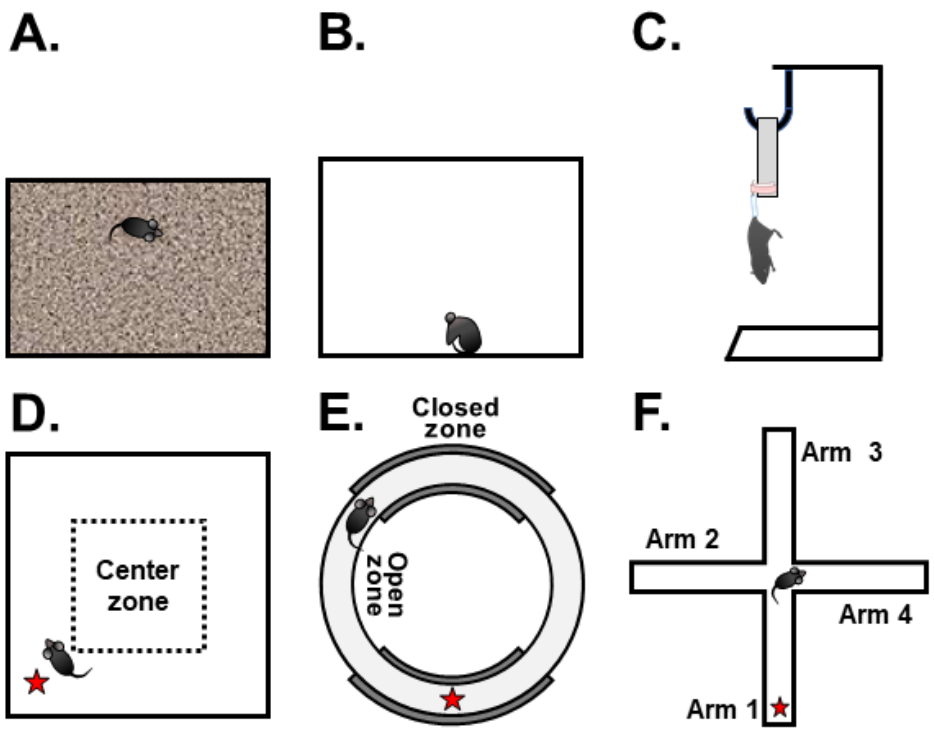


Figure 2. Behavior tests used in studies. During behavioral testing, which occurred 1- or 2-days post-challenge, (**A**) digging and (**B**) grooming activity was recorded, and frequency during the testing period was quantified. (**C**) Tail suspension test for depression-like behavior was employed. (**D**) open field recordings were used to assess anxiety-like and mobility behavior. (**E**) The Elevated-zero maze was used to measure anxiety-like behavior. (**F**) Finally, cross maze was used to assess spatial memory behavior.

Open field (OF) recordings were performed in a plexiglass enclosure (40.6-cm width × 40.6-cm depth × 38.1-cm height; San Diego Instruments, San Diego, CA, U.S.A.) with a defined 20-cm × 20-cm center zone was used (Figure 2D). Mice were individually placed in the same area of the enclosure outside of the center zone and allowed to freely explore for 10 min, and their spontaneous activities were recorded using an overhead digital video camera. The number of entries, total time spent, and average duration of visit to the center zone, and the number of fecal pellets produced during the

test duration, average speed, distance traveled, and time immobile were recorded to objectively assess anxiety-like behavior as well as overall activity levels using ANY-maze software (Stoelting Co.). The number of fecal pellets was manually counted from the video recordings by two blinded observers as a mobility-independent measure of anxiety-like behavior (Figure 2D).

Anxiety-like behavior was observed using an elevated zero maze (EZM; Stoelting Co., Wood Dale, IL, U.S.A.). Each mouse's activity on the EZM was recorded for 5 min (study 1) or 10 min (study 3). Mice were individually placed in one of the walled sections of the circular maze and allowed to explore freely. The time spent in the open zones, the number of entries into the open zones and the average duration of visit to the open zones were analyzed using ANY-maze software (Stoelting Co.) and manually validated by a blinded observer. A few mice failed to stay on the maze for the entire test duration, and they were excluded from the final analysis (Figure 2E).

A cross maze was used to evaluate spatial working memory. The maze was custom-made with white plexiglass with four identical cross-shaped arms (5-cm width × 30-cm length) with a 5-cm × 5-cm center area and 15-cm high walls based on the specifications by Kulas, et al. (2018) (Kulas et al., 2018). Mice were individually placed in one of the arms designated as the starting arm and allowed to explore the maze freely for 12 min. The sequence of arm entries was recorded by an overhead digital video camera and analyzed by a blinded observer (Figure 2F). “Alternations” was defined as the instances where the mouse successfully entered all 4 arms of the maze without reentering a previously entered arm. The percentage of alternations was calculated using the following equation (Kulas et al., 2018):

$$\%\ alternation = (\#\ of\ alternations/Total\ entries - 3) \times 100.$$

All behavior testing was performed at the University of North Dakota Behavioral Research Core Facility. Apparatuses were thoroughly cleaned with Process NPD (STERIS, Mentor, OH, U.S.A.) between tests. On days with multiple tests mice were allowed to rest for at least 1 hr between tests. The ANY-maze software (Stoelting, Co.) was used to establish all test parameters, to control video recordings, and to compute the results.

Sacrifice and Tissue Collection

Mice were euthanized via CO_2 asphyxiation followed by intracardiac perfusion with phosphate-buffered saline (PBS; pH 7.4). Cardiac blood was collected prior to perfusion to prepare serum and plasma samples for immunoglobulin and cytokine ELISAs, respectively. For plasma samples, approximately 100 µL of blood was collected in EDTA-coated Microvette tubes (Sarstedt, Inc., Newton, NC, U.S.A.) and centrifuged for 10 min at 2,000 × *g* at 4°C. For serum preparation, blood was collected in microfuge tubes and allowed to coagulate for 30 min at room temperature before centrifuged at 2,000 × *g* for 15 min at 4°C. Aliquoted serum and plasma samples were stored at −80°C until used. Brain was extracted and preserved for subsequent applications. Brains were separated into both hemispheres, the left hemisphere was preserved for histology, and the right was divided regionally, as depicted in Figure 3 for western blot analysis. In study 2, both hemispheres were dissected, as seen in Figure 3 for RNA extraction and sequencing.

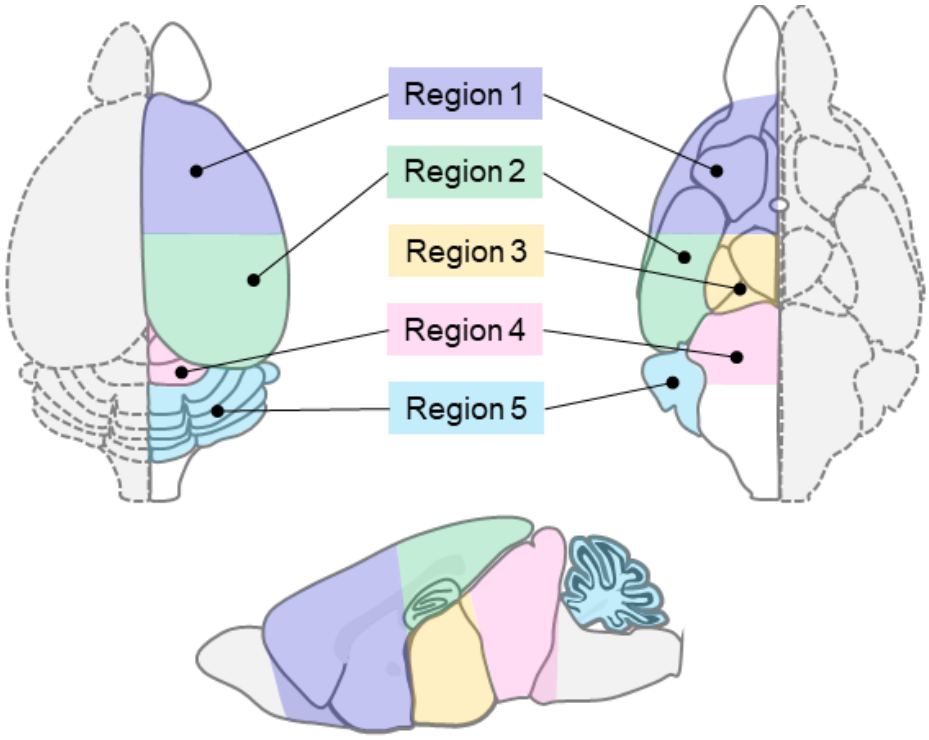


Figure 3. Regional dissection of mouse brain for biochemical analysis. Diagrams depicting the dorsal (left), ventral (right), and sagittal (bottom) views of a mouse brain. Upon collection of brain samples, the left hemisphere (L, dotted outline) was immersion-fixed in 4% PFA while the right hemisphere (R, solid outline) was subdivided into the following five regions: Region 1, rostral brain including prefrontal and frontal cortices and underlying subcortical structures (e.g., striatum); Region 2, parietotemporal cortices and the hippocampus; Region 3, the thalamus and hypothalamus, Region 4, the midbrain; and Region 5, the cerebellum.

Enzyme-Linked Immunosorbent Assays (ELISA)

In studies 1 and 2, the amount of antigen-specific IgE and IgG1 antibodies present in sera was quantified using ELISA. The wells of the ELISA plate were coated with 20 μg/mL BLG in a 100 mM sodium carbonate/bicarbonate buffer overnight at 4°C, washed thoroughly, and blocked with PBS containing 0.05% Tween-20 and fetal bovine serum (Assay Buffer, eBioscience ELISA Support Pack Plus # BMS414, Thermo Fisher Scientific, Waltham, MA, U.S.A.). Sera isolated from the terminal blood of mice were diluted 1:1 for IgE or 1:50 for IgG1 detection with Assay Buffer before adding to the antigen-coated wells. The plate was incubated for 12 hrs at 4°C, and BLG-specific IgE was detected with biotinylated anti-mouse IgE (used at 1:1,000, #13-5992-81, ThermoFisher Scientific) or anti-mouse IgG1 (used at 1:1,000, #13-4015-82, ThermoFisher Scientific) followed by avidin-HRP and TMB (3,3′,5,5′-tetramethylbenzidine) according to the manufacturer's instructions. The plate was read at 450 nm on a Biotek ELx 800 microplate reader using Gen5 v3.02 software (Biotek Instruments, Winooski, VT, U.S.A.).

In study 3, serum samples from mice were first diluted to 1:40 to adsorb IgG using magnetic protein G beads, and resulting supernatants were used for BLG-specific IgE detection. Bead-bound IgG was eluted in 50 mM glycine (pH 2.8) for 2 min at room temperature, neutralized with 1 M Tris buffer (pH 7.5), and used to detect BLG-specific IgG1 and IgG2a. Samples were placed in the allergen-coated wells and incubated overnight at 4°C. BLG-specific immunoglobulins were detected by respective anti-mouse secondary antibodies followed by avidin-HRP and using TMB as the substrate. Reactions

were terminated by adding 2 N H_2SO_4 prior to reading at 450 nm on a Biotek ELx 800 microplate reader with Gen5 software (Biotek Instruments).

The amount of TNFα in the midbrain samples was quantified using TNFα DuoSet® ELISA (#DY410, R&D Systems, Minneapolis, MN, U.S.A.) according to the manufacturer's protocol. Briefly, an ELISA plate (#2580, Corning EIA/RIA 8-Well Strips) was coated with TNFα capture antibody overnight at room temperature. After washing and blocking the wells, protein extract (200 ng/100 µL/well) was placed in duplicates and incubated for 2 hrs at room temperature. TNFα in the samples was visualized by sequentially incubating the wells with the detection antibody and streptavidin-HRP. The substrate reaction was allowed to occur for 20 min before termination, and the plate was read as described above. The mean of the duplicate values from each sample was taken, and TNFα concentration was calculated from the standard curve.

Tissue Preparation for Histology

The left hemisphere of the brain and the ileum were immersion fixed in 4% paraformaldehyde for 24 hrs. Following fixation, tissues were dehydrated in increasingly high sucrose-containing PBS solutions. First, immediately following fixation, tissues were places 15% sucrose for 24 hrs then placed in 30% for 24 hrs or until embedded. Fixed ileal tissues were placed in either optimal cutting temperature (OCT; study 2; #4583, Sakura Finetek, Torrance, CA, U.S.A.) solution or gelatin (study 1), and brain tissues were embedded in gelatin. Gelatin embedded ilea and brains were then processed

as explained in Nagamoto-Combs, et al. (2016) (Nagamoto-Combs et al., 2016) and frozen sectioned at 14 and 40 μm, respectively.

Immunohistochemistry

Tissue sections were treated with 0.3% peroxidase and blocked in PBS containing 0.5% bovine serum albumin and 5% normal goat serum (#16210072, ThermoFisher Scientific) and incubated with a primary antibody against rabbit anti-mouse occludin (used at 1:100, #711500, ThermoFisher Scientific), glial fibrillary acidic protein (GFAP; used at 1:1,000, #12389S, Cell Signaling Technology, Boston, MA, U.S.A.), Iba1 (used at 1:1000, #019-19741, Wako Chemicals, Richmond, VA, U.S.A.), myelin basic protein (MBP; used at 1:500, #HPA049222, MilliporeSigma), or IgG (used at 1:500, #13-4013-85, ThermoFisher Scientific) for 12–48 hrs at 4°C. Tissues were subsequently incubated in anti-rabbit IgG (used at 1:2,000, #PK-6101, Vector Laboratories, Burlingame, CA, U.S.A.) or mouse-adsorbed rabbit anti-rat IgG (used at 1:100, #BA-4001, Vector Labs) antibody. Immunoreactivity was visualized with Vector Elite ABC kit (#PK-6101, Vector Labs) with VIP as the chromogen (#SK-4600, Vector Labs).

RNA Extraction and Reverse Transcriptase Quantitative PCR

Ileal and brain tissue samples were homogenized in TRIzol solution (#15596018, Thermo Fisher Scientific) using 5-mm stainless steel beads in a Bullet Blender Storm 24 (Next Advance, Troy, NY, U.S.A.) set to speed 6 for 3 min with 30-sec intervals. Total RNA was extracted and purified by ethanol precipitation according to the manufacturer's instructions. The amount of RNA was quantified using Nanodrop One (Thermo Fisher Scientific), and 1 μg of total RNA was used to synthesize cDNA with an iScript™ cDNA

Synthesis Kit (#1708891, Bio-Rad Laboratories, Hercules, CA, U.S.A.) by priming at 25°C for 5 min, reverse transcription at 46°C for 20 min, and inactivation at 95°C for 1 min. Quantitative PCR reactions were performed with 100 ng cDNA and specific primer sets for murine *Tnfα* (qMmuCED0004141, Bio-Rad), occludin (*Ocln*; fwd: 5′-AAAGCAAGTTAAGGGATCTG-3′; Rev: 5′-TGGCATCTCTCTAAGGTTTC-3′, MilliporeSigma), phosphatidylserine decarboxylase pseudogene 1 (*Pisd-ps1*; fwd: 5′-ACGAGTTTGCTGTCATGTGC-3′; Rev: 5′-TCAGTCATGTTCACCCCAAA-3′, MilliporeSigma), myocardial infarction associated transcript (*Miat*; fwd: 5′-CCCACATCTCTTTGCTTGAGTCC-3′; Rev: 5′- GCTCTTTGTGCCCAGCTCTTAAC-3′, MilliporeSigma), maternally expressed 3 (*Meg3*; fwd: 5′-ACATGCTGGACCCAAGACTC-3′; Rev: 5′- CCTGAGCCCATTTCACAGAT-3′, MilliporeSigma), Heat shock protein h1 (*Hsph1*; fwd: 5′-CAGGTACAAACTGATGGTCAACA-3′; Rev: 5′-TGAGGTAAGTTCAGGTGAAGGG-3′, MilliporeSigma), Transferrin (*Trf*; fwd: 5′-TGGGGGTTGGGTGTACGAT-3′; Rev: 5′- AGCGTAGTAGTAGGTCTGTGG-3′, MilliporeSigma), RNA binding motif protein 3 (*Rbm3*; fwd: 5′-CTTCGTAGGAGGGCTCAACTT-3′; Rev: 5′- CTCCCGGTCCTTGACAACAAC-3′, MilliporeSigma), ribosomal protein S18 (*Rps18*; qMmuCED0045430, Bio-Rad) or glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*; qMmuCED0027497, Bio-Rad) using iTaq™ Universal SYBR® Green Supermix (#1725120, Bio-Rad) on a C1000 Touch Thermo Cycler (Bio-Rad) for 40 cycles (denaturing at 95°C for 15 sec, annealing at 60°C for 30 sec, and extension at 72°C for 30 sec). The resulting Cq values were calculated using Bio-Rad CFX Manager Software version 3.1. Target gene ΔΔCq values

were calculated by normalizing target Cq values to the Cq values of reference genes *Rps18* or *Gapdh* in the brain and ileum, respectively.

Western Blot Analysis

Total proteins from each isolated region of the right brain hemisphere were extracted in RIPA buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM Na_3VO_4 10 mM sodium fluoride, 1 mM EDTA, 1 mM EGTA, 0.2 mM phenylmethylsulfonyl fluoride, 1% Triton X-100, 0.1% SDS, and 0.5% deoxycholate) and quantified using the Bradford method (Bradford, 1976). Western blotting was carried out with 25 µg of protein samples resolved on 15% SDS-polyacrylamide gels. Resolved proteins were transferred onto PVDF membranes and detected with a primary antibody against GFAP (used at 1:1,000, #12389S, Cell Signaling Technology), cyclooxygenase 2 (COX-2; used at 1:1,000, #sc-1745, Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) or GAPDH (used at 1:1,000, sc-32233, Santa Cruz Biotechnology) overnight at 4°C or 2 hrs at room temperature with gentle rocking. The membranes were subsequently incubated in an appropriate HRP-conjugated secondary antibody (Santa Cruz Biotechnology). Target proteins were visualized using Amersham ECL Prime Western Blotting Detection Reagent (#RPN2232, GE Healthcare, Pittsburgh, PA, U.S.A.) on an Aplegen Omega Lum G Gel Documentation System (Gel Company, Inc., San Francisco, CA, U.S.A.). After the detection of chemiluminescence signal, PVDF membranes were treated with 0.2 N sodium hydroxide for 10 min at room temperature with gentle agitation to remove the antibodies and re-probed for another target protein. The levels of proteins were quantitated from the captured image using LI-COR Image Studio Lite Software 5.0 (LI-

COR Biosciences, Lincoln, NE, U.S.A.) and normalized to the amount of GAPDH detected from the same PVDF membrane.

Gut Permeability Assay

In study 2, mice were orally given 4 kDa fluorescein isothiocyanate (FITC) labeled dextran prior to sacrifice. The volume of FITC-dextran given was equal to 484 μg/g mouse weight. Five hours following FITC-dextran gavage, the mice were sacrificed, blood was collected, and serum separated. Serum was placed on a 96-well plate, and FITC emission wavelength of 528 nm was read after excitation at 485 nm. The resulting emission was converted into a concentration of FITC based upon signal detected from standards.

Cytokine ELISA Array

Plasma samples prepared from terminal blood were used to profile cytokines and chemokines using Quantibody® Mouse Cytokine Array Q5 Kit. Samples were first diluted to 1:5 before assayed using the multiplex kit according to the manufacturer's instructions. Upon completing the assay procedure, the slides were sent to RayBiotech Array Scanning and Analysis Services to quantify the fluorescence signals. The resulting fluorescence signal values were analyzed using RayBiotech Q-Analyzer®, an array-specific Microsoft Excel-based software tool (https://www.raybiotech.com/files/analysis-tools/QAM-CYT-5.xls).

RNA-Sequencing and Ingenuity Pathway Analysis

The quality and quantity of extracted brain RNA were assessed using Qubit 2.0 Fluorometer and Agilent 2100 Bioanalyzer at UND Epigenetics Bioinformatics Core

Facility. Three highest-quality brain samples were chosen per treatment group and sequenced by Novogene Corporation (Chula Vista, CA, U.S.A) on an Illumina HiSeq X Ten sequencer (San Diego, CA, U.S.A.) with 150 bp paired-end reads. Bioinformatic analysis was performed by UND Epigenetics Bioinformatics Core Facility, identifying brain region-specific differentially expressed genes.

Pathway analysis was performed on regional differentially expressed genes using Ingenuity Pathway Analysis software (Qiagen Inc., Germantown, MD, U.S.A.). BLG/Sham Log_2(fold expression) of differentially expressed genes (p-value < 0.05) were placed into analysis software and evaluated for brain-specific pathways to identify upstream regulators, canonical pathways, and disease states.

Microbial DNA Extraction

After 1 week of acclimation with the whey-free diet, each mouse was individually placed in a cage until stool samples were collected and returned to its home cage with original cage mates. Fecal pellets from each mouse were collected in an autoclaved microfuge tube using sterile forceps and placed on ice until pellets were harvested from all mice. Stool samples were frozen stored at −80°C until used for microbial DNA extraction. Additional fecal samples were collected in the same manner immediately prior to euthanasia to assess post-sensitization changes in fecal microbiomes. To compare fecal microbiome profiles, microbial DNA was extracted from stool samples using ZymoBIOMICS DNA kit (Zymo Research, Irvine, CA, U.S.A.)

16s Sequencing and Microbial Pathway Analysis using Kyoto Encyclopedia of Genes and Genomes

Extracted microbial DNA was sent to the Genome Technology Access Center at Washington University (St. Louis, MO, U.S.A.) for 16S ribosomal RNA gene sequencing. Sample library preparation for 8 hypervariable regions of the 16S gene was performed with Fluidigm Juno LP 192.24 IFC system. Sequencing was carried out on a HiSeq 3000 with approximately 11M 150 bp paired-end reads, yielding an average of 44,277 reads per sample. The raw FASTQ files were assessed for quality by DADA2. The forward and reverse reads were truncated using filterAndTrim function of DADA2 package at 140 and 130 base position, respectively. After dereplication by DADA2, filtered paired-end reads were merged, and a quality-aware correction model was used to remove noise and chimeras to call final amplicon sequence variants (ASVs) (Callahan et al., 2017). ASVs were classified taxonomically based on the SILVA database (version 132, https://zenodo.org/record/1172783#.X3zfvy2ZOL8), and the ASVs that were not assigned to phyla or assigned to non-bacterial kingdoms by the phyloseq R package (version 1.32.0) (McMurdie and Holmes, 2013) were removed. Furthermore, the ASVs with prevalence < 9 and total abundance < 222 (lower quartile) were excluded from downstream analysis. Taxonomy classification for trimmed reads was performed by Kraken 2 (version 2.0.8-beta) (Wood et al., 2019) based on MiniKraken2_v1 database (version 201904). Differential species were screened out by the DESeq2 package (version 1.28.1).

The alpha diversity metrics were analyzed using the estimate_richness function. Proportionally normalized data was used for Bray-Curtis Principal Coordinates Analysis

(PCoA) to reveal differences between experimental groups. The 'Adonis' feature from the vegan (version 2.5-6) R package was used to assess whether sample grouping by metadata factor accounted for inter-sample differences (Oksaren et al., 2020). The DESeq2 package (version 1.28.1) was used to identify significant differences in ASVs between groups (Love et al., 2014). Differential ASVs were then subjected to KEGG pathway profile analysis using Tax4Fun2 R package (version 1.1.5) (Wemheuer et al., 2020) with 'Ref99NR' database mode and Tax4Fun2_ReferenceData_v1.1. KEGG pathways with adjusted p-value < 0.05 were considered significant after multiple testing correction.

qPCR Quantification of *Akkermansia muciniphila*

The abundance of *A. muciniphila* at pre- and post-sensitization was detected from 100 ng of microbial DNA using quantitative PCR (qPCR). The species-specific primers, S-St-Muc-1129-a-a-20: 5'-CAG CAC GTG AAG GTG GGG AC-3' and S-St-Muc-1437-a-A-20: 5'- CCT TGC GGT TGG CTT CAG AT-3' (Collado et al., 2007) and iTaq™ Universal SYBR® Green Supermix (Bio-Rad) were used to perform qPCR on a C1000 Touch Thermocycler (Bio-Rad) for 40 cycles (denaturing at 95°C for 15 sec, annealing at 55°C for 30 sec, and extension at 72°C for 30 sec). Cq values were computed using Bio-Rad CFX Manager 3.1, and the amount of *A. muciniphila* was expressed as 2^{-Cq}.

Statistical Analysis

All statistical analyses, except the microbiome analysis, were performed using GraphPad Prism 9 software (GraphPad Software, Inc., San Diego, CA, U.S.A.). Statistical significance of the differences between sham and BLG groups was

independently analyzed for male and female groups using unpaired t-tests. Welch's correction was used when sample sizes varied, and Mann-Whitney test was employed where normal distribution of data values was not observed. Differences between strain-matched sham and BLG groups and between treatment-matched two strains were compared independently among male and female groups using two-way ANOVA with Fisher's least significant difference (LSD) test, when multiple comparisons were necessary, unless specified in the figure legends. The ROUT method (Q=1%) was used to identify outliers in a group when appropriate, and the values were removed from the final results. A p value less than 0.05 ($p < 0.05$) was considered statistically significant.

CHAPTER III

RESULTS

Study 1 – Astrogliosis Associated with Behavioral Abnormality in a Non-anaphylactic Mouse Model of Cow's Milk Allergy

Contents of this chapter were originally published in Frontiers in Cellular Neuroscience (Smith et al., 2019)

Introduction

Behavioral and emotional disorders, such as anxiety, depression, attention-deficit hyperactivity disorder (ADHD), obsessive-compulsive disorder, and autism, are major mental health problems that could severely affect quality of life. In the United States alone, ~20% of adolescents and adults are reported to have experienced mental disorders in 2016 (Center for Behavioral Health Statistics and Quality). The actual number of people who suffer from these conditions is expected to be greater than reported, considering that these disorders often go undiagnosed due to unwillingness of patients to disclose their conditions or failure of their caregivers to recognize the symptoms (Glazier et al., 2015; Hirschtritt et al., 2017; Klik et al., 2018). Even among diagnosed patients who seek treatments, some are resistant to conventional pharmacological and psychotherapeutic interventions, requiring increased dosage of medications and/or more aggressive treatments such as electroconvulsive therapy and neurostimulation (Al-Harbi, 2012; Hirschtritt et al., 2017). Nonetheless, not all patients benefit from these treatments,

signifying the need for alternative intervention approaches for these debilitating conditions.

Interestingly, certain dietary items have been long suspected to trigger or exacerbate emotional and behavioral symptoms (Crayton, 1986), suggesting a potential role of food allergy/hypersensitivity (FAH) in the etiology of neuropsychiatric conditions. While many clinical cohort studies have reported that significant behavioral comorbidities exist among individuals with FAH (Addolorato et al., 1998; Parker and Watkins, 2002; Costa-Pinto and Basso, 2012; Shanahan et al., 2014; Ferro et al., 2016), the contributory role of diet in neuropsychiatric disorders has been controversial due to insufficient pathophysiological evidence and inconsistent results across studies. In order to determine the causative role of FAH in behavioral changes without genetic, dietary, and environmental variables commonly associated with human cohorts, we utilized a mouse model of cow's milk allergy (CMA) and examined behavioral changes and pathophysiology in the gut and brain mediated by FAH.

To observe CMA-mediated changes in typical innate activities of mice, a non-anaphylactic mouse model of CMA was previously established by orally sensitizing the C57BL/6J strain of mice with a whey protein (WP) mixture and cholera toxin (CT) as an adjuvant (Germundson et al., 2018). These sensitized mice generally exhibited mild to no anaphylaxis upon WP challenge, allowing a series of behavioral assessments to be performed the next day. In this study, we limited the allergen to β-lactoglobulin (BLG; Bos d 5), in order to isolate the effect of this major whey allergen, which is absent in human breast milk (Malacarne et al., 2002). We report that BLG-sensitized male mice displayed anxiety- and depression-like behavior similar to the mice sensitized with the

WP mixture. Moreover, we found astrocytic hypertrophy in the ventral midbrain of the BLG-sensitized brain, particularly near the blood vessels, resembling the perivascular "barriers" or "cuffs" described in mouse models of experimental autoimmune encephalitis (EAE) (Voskuhl et al., 2009; Sofroniew and Vinters, 2010). These results indicated that oral BLG sensitization of otherwise healthy mice results in region-specific perivascular astrogliosis, likely modifying the functional property of the blood brain barrier.

BLG Sensitization of C57BL/6J Mice Results in Increased Serum Levels of Allergen-specific IgE and IgG1 in Male Mice Without Eliciting Obvious Signs of Anaphylaxis After BLG Challenge

To monitor the overall health and steady growth of the experimental mice, their body weights were recorded during the sensitization protocol. The average body weights of mice in each group before the initiation of sensitization (Week 1) and at the time of allergen challenges (Week 6 and 7) were compared between the sex-matched treatment groups (Figure 4A). No significant differences were found in the body weights between the groups at any of the time points examined, suggesting that BLG-sensitized mice had comparable growth to the sham mice.

A week after the 5 weekly sensitization procedures, all mice underwent a BLG challenge in Week 6 for the assessment of their physical responses to allergen re-exposure. No obvious signs of anaphylaxis were exhibited by male or female mice from both of the treatment groups at 30 min post-challenge. This lack of physical reactions to the allergen was observed again after the second challenge in Week 7.

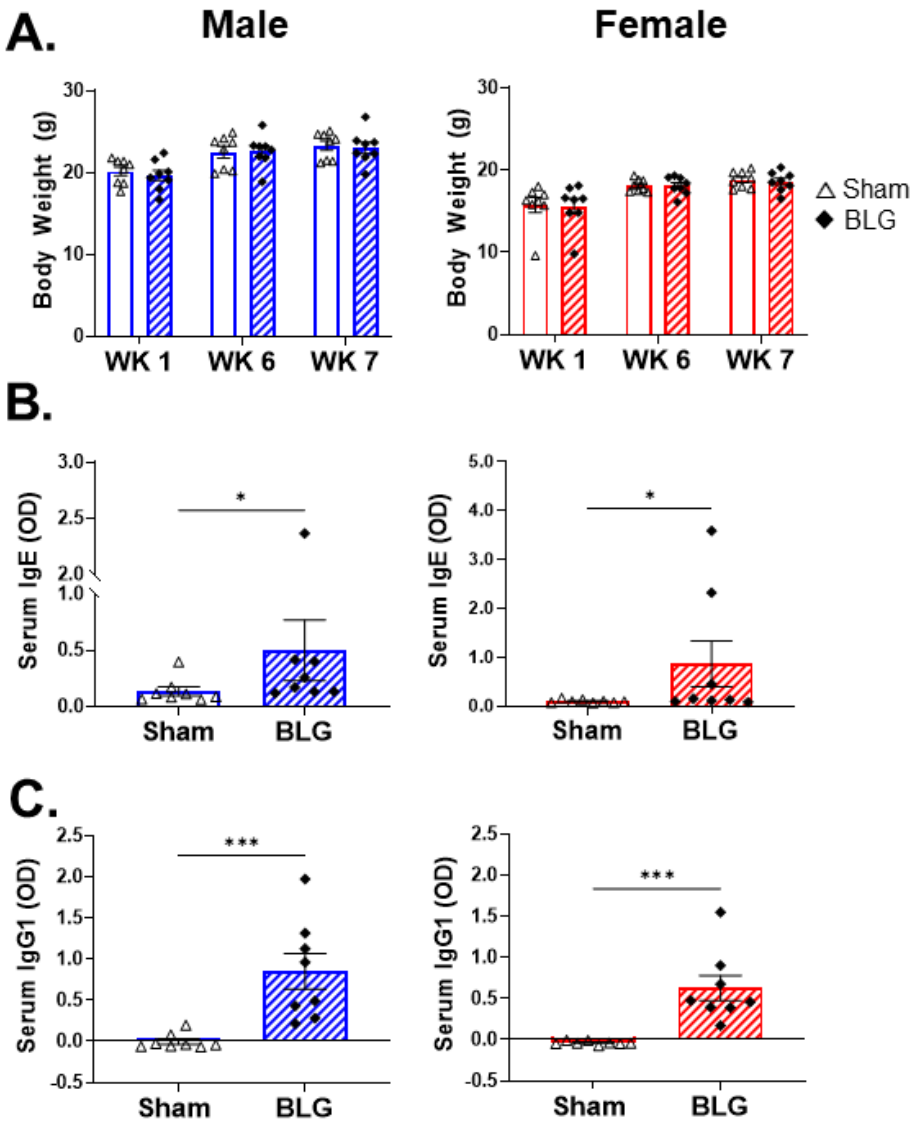


Figure 4. Physical growth and adaptive immunity development during BLG sensitization. (**A**) Weights of mice were recorded during Weeks 1, 6, and 7 of sensitization to assess potential impact of the sensitization regime on overall health and growth. (**B**, **C**) Serum isolated from the terminal blood was used to quantify the levels of BLG-specific IgE (**B**) or IgG1 (**C**) using ELISA. Values shown in the graphs indicate the group average ± SEM, $*p < 0.05$; $***p < 0.001$. (Mann-Whitney test), male sham: $n = 8$; male BLG: $n = 8$; female sham: $n = 8$; female BLG: $n = 8$.

In order to ensure that the BLG-sensitization protocol successfully induced acquired immunity in our mouse model, we next determined the levels of serum BLG-specific IgE and IgG1 using ELISA (Figure 4B, C, respectively). While the serum levels of BLG-specific IgE were comparable among the male and female sham groups, both of the BLG sensitized groups showed wider ranges of BLG-specific IgE levels. The serum samples from a few mice in each group contained much greater BLG-specific IgE than the others within the group (Figure 4B). There were modest but significant increases in the average levels of BLG-specific IgE in both sensitized male and female mice compared to their respective sham groups (male sham: 0.14 ± 0.04; male BLG: 0.5 ± 0.3; female sham: 0.10 ± 0.01; female BLG: 0.9 ± 0.5; $n = 8$ in all groups, $p < 0.05$, Mann-Whitney test). When the extreme values were identified as outliers by GraphPad Prism software and removed from the analysis, the statistical significance of the BLG-induced IgE levels increased to $p < 0.01$ for male mice (male sham: 0.10 ± 0.02, $n = 7$; male BLG: 0.23 ± 0.05, $n = 7$; one outlier from each group was removed from the analysis [sham, 0.40; BLG, 2.37]). In contrast, removal of outliers from the female groups resulted in the loss of statistical significance for female groups [female sham: 0.10 ± 0.01, $n = 8$; female BLG: 0.17 ± 0.06, $n = 6$; two outliers removed from the analysis of the BLG group [3.58, 2.32]. The analysis excluding the outliers is shown in Supplemental Figure 1. Similarly, BLG-specific IgG1 levels were also elevated in the sensitized mice for both sexes with less variability than IgE (Figure 4C). These results indicated that the BLG-sensitization procedure elicited acquired immunity toward BLG with elevated antigen-specific IgE and IgG1 in both male and female mice. Although a subset of sensitized mice showed greater

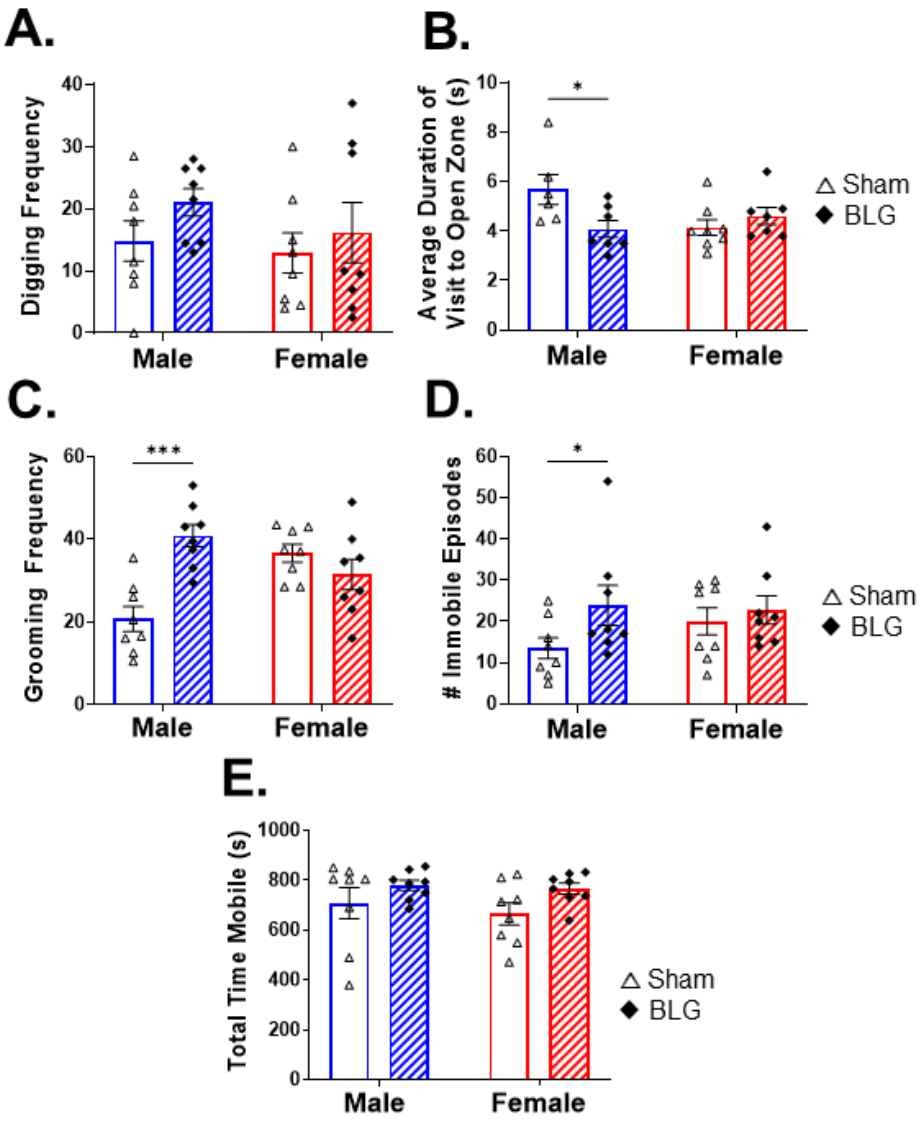
degrees of antibody productions, their apparent physical health was not visibly affected, and the allergen challenge did not result in anaphylaxis.

BLG Sensitization Resulted in Anxiety- and Depression-Like Behavioral Changes in Male C57BL/6J Mice

BLG-mediated changes in mouse behaviors were examined using 4 different behavioral tests. The digging behavior observation and EZM were performed after the first challenge while grooming behavior observation and TST were carried out after the second challenge (see the Methods section). The frequency of digging activity within the 10-min observation period was 15 ± 3 in male sham, 21 ± 2 in male BLG-sensitized, 13 ± 3 in female sham, and 16 ± 5 in female BLG-sensitized mice (n = 8 per group, Figure 5A). Although there was a trend toward increased digging activity in male BLG-mice compared to their sex-matched sham, the difference did not reach a statistical significance (p = 0.1). However, the EZM showed a significant decrease in the average duration of visit to open zone in male mice (sham: 5.7 ± 0.6, n = 6; BLG: 4.1 ± 0.3; n = 7; $p < 0.05$) and not in female (sham 4.2 ± 0.3, n = 8; BLG: 4.6 ± 0.3; n = 7; Figure 5B). Further surveillance of the test recordings revealed that the mice with a shorter duration of visit to open zones often did not walk through the open zone to the other closed zone, but they briefly surveyed the entry to the open zone and returned to the original closed zone (See Supplemental Video 1 + 2). In addition, the analysis of grooming behavior after the second BLG challenge indicated that BLG-sensitized male mice groomed themselves more often than their sham counterpart (sham: 20 ± 3, n = 8; BLG: 41 ± 3; n = 8; $p < 0.001$) while no sensitization-dependent differences were observed between the female groups (sham: 36 ± 2, n = 8; BLG: 31 ± 4; n = 8), indicating

that only male BLG-sensitized mice displayed more anxiety-like behavior than sham mice (Figure 5C). Similarly with the TST (Figure 5D), only male BLG-mice exhibited more depression-like behavior than the sham with greater numbers of immobile episodes during the 6-min testing period (sham: 14 ± 3, $n = 8$; BLG: 24 ± 5; $n = 8$; $p < 0.05$) while female sensitized mice did not (sham: 20 ± 3, $n = 8$; BLG: 23 ± 3; $n = 8$). To assure that the observed behavioral differences in the sensitized mice were not due to lethargy-related immobility, the total time in seconds mice were mobile during their digging tests were compared between the groups (Figure 5E). There were no obvious differences in the total time mobile among male and female sham and BLG mice, indicating that the changes in the behavioral parameters observed with the male BLG-sensitized mice did not result from physical or ambulatory difficulties. These results demonstrated that the male mice were susceptible to behavioral alterations upon BLG sensitization, even though they did not exhibit apparent anaphylactic reactions when challenged with the allergen.

Figure 5. Assessments of anxiety- or depression-like behavior after BLG challenge. Behavioral tests were performed at 1- and 2-day post-challenge. (**A**) Digging frequency was quantified by two observers who were unaware of the experimental conditions. Either the presence (1 point) or absence (0 points) of digging behavior was recorded in every 10-sec interval during the 10-min test period. The total points scored by the two observers were averaged and used as the final score for each mouse. (**B**) For EZM test, the average duration each mouse spent within the open zone per visit was computed by ANYmaze software and later validated by an observer. (**C**) Grooming frequency was quantified by two observers for scoring either the presence (1 point) or absence (0 points) of grooming behavior as described for the digging frequency scoring. (**D**) For TST, the number of immobility episodes was used as the measure of the mice's helpless behavior that reflected their depression-like state. (**E**) Total time mobile was also computed from the recordings during the digging behavior to verify their motility to distinguish their immobility from lethargy. Values shown in the bar graphs indicate the group average ± SEM, $*p < 0.05$, $***p < 0.001$ (unpaired t-tests) $n = 7–8$.


A.
Digging Frequency
40
30
20
10
0
Male
Female
B.
Average Duration of Visit to Open Zone (s)
10
8
6
4
2
0
*
Male
Female
△ Sham
◆ BLG
C.
Grooming Frequency
60
40
20
0
***
Male
Female
D.
Immobile Episodes
60
50
40
30
20
10
0
*
Male
Female
△ Sham
◆ BLG
E.
Total Time Mobile (s)
1000
800
600
400
200
0
Male
Female
△ Sham
◆ BLG

BLG Sensitization Altered the Levels of Tight Junction Protein and the Expression of Proinflammatory Cytokine in the Small Intestine

Next, we sought to identify changes in different brain regions that might contribute to the observed sex-dependent behavioral abnormality. Since BLG-mediated behavioral changes were only observed in male mice, we focused our further analyses on male animals. Allergens that paracellularly enter intestinal mucosa through compromised epithelial tight junction barriers may be recognized as pathogens by antigen presenting cells (APCs) and initiate immune responses. To examine whether BLG sensitization resulted in decreased barrier integrity, the levels of a tight junction protein, occludin, were examined in the intestinal tissue. Immunohistochemical assessment of the ileum from the male sham (Figures 6A,a) and BLG-sensitized mice (Figures 6B,b) showed that there was decreased staining in the villi of the latter group. This decreased immunoreactivity for occludin was likely a result of post-translational regulation since the amount of occludin transcripts in the ileal tissue from BLG-sensitized mice did not differ from the sham mice when determined using RT-qPCR (Figure 6C).

Aberrant paracellular infiltration of allergens could trigger inflammatory responses by intestinal immune cells. Thus, we also investigated the inflammatory status of the gut mucosa by determining the amount of a proinflammatory cytokine, TNFα. An RT-qPCR assay indicated that there was a modest but significant increase in TNFα mRNA in the ileum (Figure 6D), signifying the presence of proinflammatory events at the site of allergen insult.

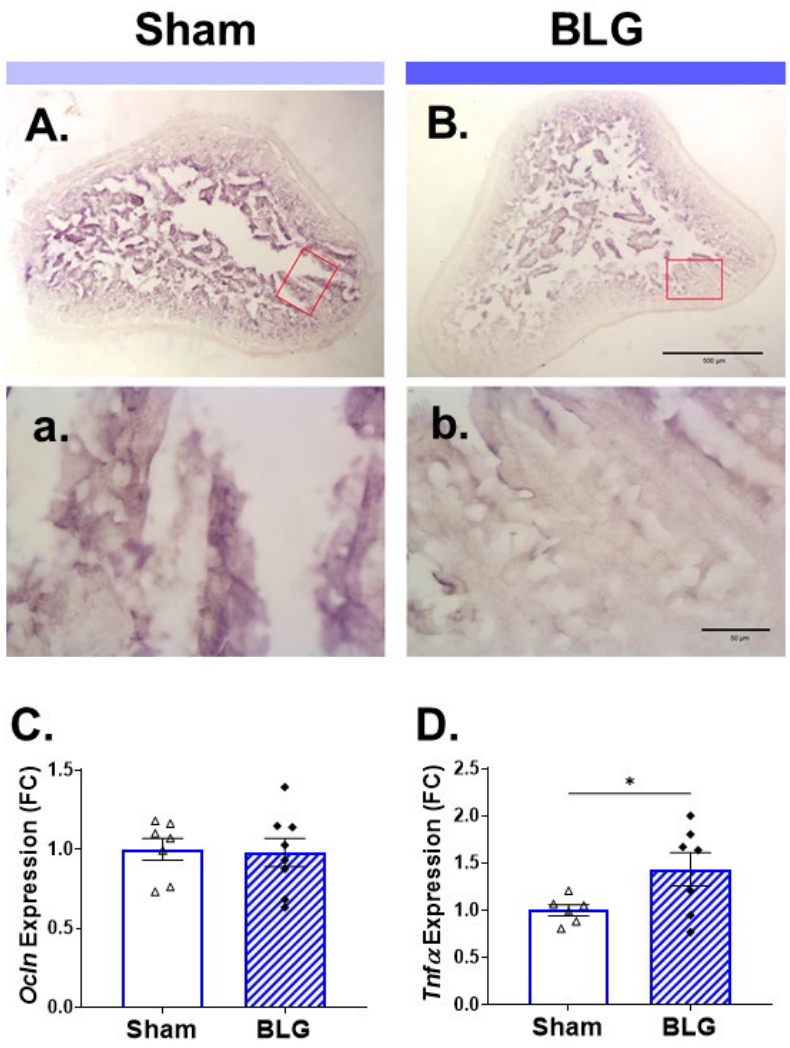


Figure 6. Immunohistochemical detection of occludin and the RT-qPCR assays for *Ocln* and *Tnfα* expression in the ileum of male sham and BLG mice . Ileal sections (14 μm) from male sham (**A**, **a**) and BLG (**B**, **b**) mice were immunostained for occludin. The red rectangles in panels (**A**, **B**) indicate where the respective higher magnification images, (**a**) and (**b**), were taken. The low-magnification (**A**, **B**) and high-magnification (**a**, **b**) images were taken with a 4X and 40X objectives, respectively. Scale bars: 500 μm for (**A**) and (**B**); 50 μm for (**a**) and (**b**). The expression levels of *Ocln* (**C**) and *Tnfα* (**D**) in the ileal tissue samples were also quantitated using RT-qPCR assay. The Cq values for *Ocln* and *Tnfα* were normalized to the Cq values of *Gapdh* (ΔCq) to calculate the expression values ($\Delta Cq = 2^{-\Delta Cq}$). Values shown in the bar graphs are expressed as the fold change (ΔΔCq) ± SEM. *$p < 0.05$ (unpaired t-test with Welch's correction), $n = 6–8$.

GFAP-Immunoreactive Astrocytes Were Hypertrophic in the Midbrain Region of the BLG-Sensitized Mice

Under the hypothesis that glia cells could respond to inflammatory mediators from the intestine and elicit neuroinflammation that would ultimately result in altered behavior, brain tissues from sham and BLG-sensitized male mice were immunostained for astrocyte and microglia markers, GFAP and Iba1, respectively. Iba1-immunopositive cells were observed throughout the brain sections although we did not observe noticeable differences between sham and BLG-sensitized mice (not shown). GFAP-stained astrocytes were also found ubiquitously in the brain, but they were more localized to specific regions such as within the white matter. In midbrain sections, the majority of astrocytes were found within the substantia nigra pars reticulata (Figure 7). GFAP-positive cells were abundantly present in both sham (Figure 7A) and BLG-sensitized (Figure 7B) mice. Interestingly, astrocytes in this area of BLG-sensitized mouse brain appeared darker and their processes seemed greater in number and thickness (Figure 7b', b"). Perivascular astrocytes were notably different, with apparently increased density of GFAP-positive end-feet contacting the vascular wall (arrowheads in Figure 7b'). These observations provided evidence for glial response, at least by astrocytes in the midbrain regions, in the central nervous system of BLG-sensitized mice.

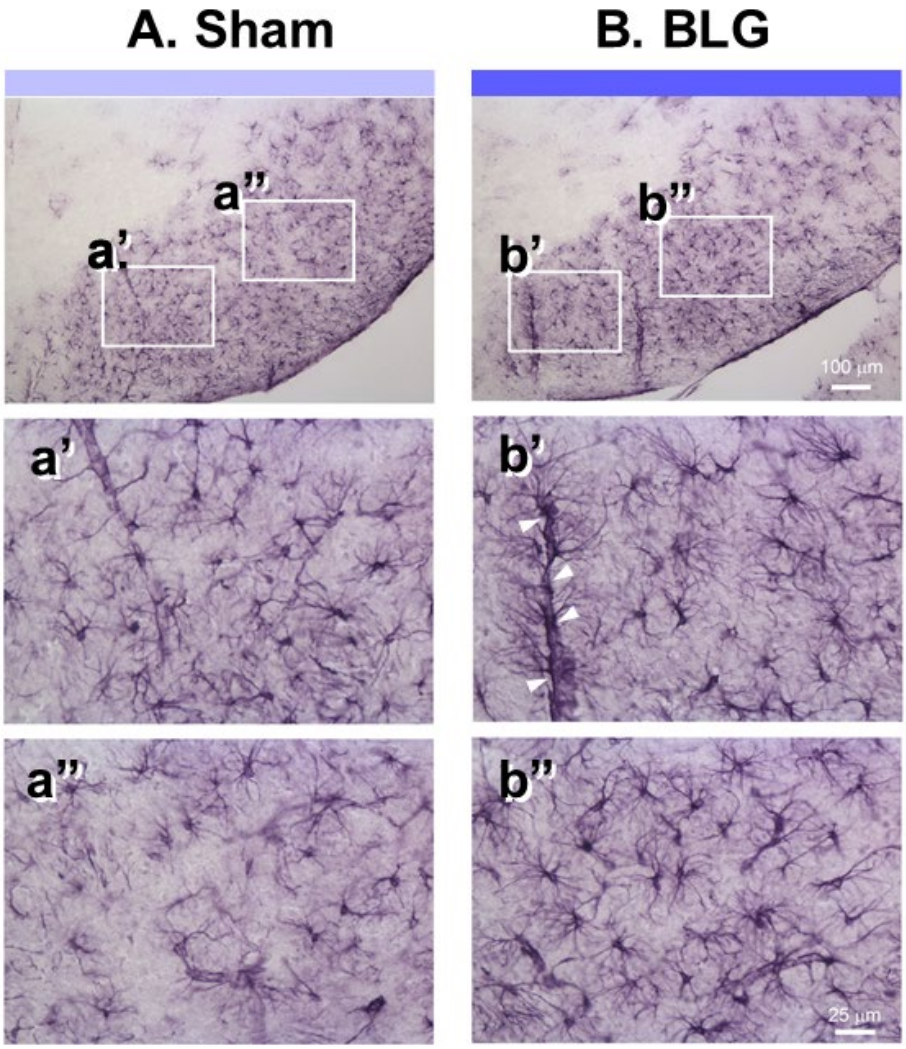
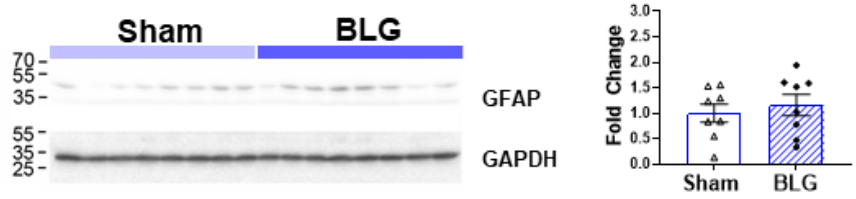


Figure 7. GFAP immunoreactivity in the midbrain of sham and BLG-sensitized mice. GFAP-positive astrocytes were identified by immunohistochemically staining brain sections (40 µm). Representative midbrain sections from sham (**A**, **a'**, **a”**) and BLG-sensitized (**B**, **b'**, **b”**) male mice are shown. The white rectangles in (**A**, **B**) indicate where the respective higher magnification images **a'**–**b”** were taken. The open arrowheads indicate dense GFAP-immunoreactive astrocyte end-feet along the blood vessel. The low-magnification (**A**, **B**) and high-magnification (**a'-b”**) images taken with a 10X and 40X objectives, respectively. Scale bars: 100 µm for (**A**) and (**B**); 25 µm for **a'**–**b”**.

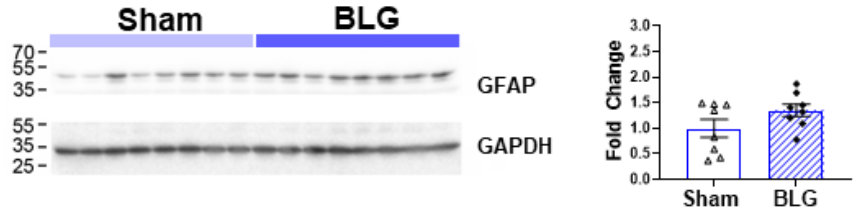
In order to verify our immunohistological observations, western blot analysis was performed using protein extracts from different brain regions (Figure 8, see Figure 3 for the division of the regions). The level of GFAP was slightly elevated in the Region 2 (parietotemporal cortices and hippocampus) and Region 3 (thalamus and hypothalamus) of the BLG-sensitized mice, although the difference was not statistically significant (Region 2: 1.4 ± 0.2 fold, $p = 0.1$; Region 3: 1.5 ± 0.3 fold, $p = 0.1$). However, in Region 4 containing the midbrain and rostral brainstem, the difference in GFAP levels between the two groups of mice was significant with a 1.6 ± 0.2 fold increase in the sensitized mice ($p < 0.001$). This result indicated that BLG sensitization resulted in upregulation of GFAP in this region, corroborating our immunohistological observation of hypertrophic astrocytes in the same region. As an additional marker of proinflammatory change, we next examined protein levels of COX-2 in the various brain regions (Figure 9). Exactly as observed when examining GFAP levels, a significant increase in COX-2 protein levels in sensitized mouse brains was noted only in the midbrain and rostral brainstem samples (Region 4).

Figure 8. Western blot analysis of GFAP in the isolated five brain regions. Soluble proteins isolated from the 5 regions were resolved on discontinuous 15% SDS-polyacrylamide gels for western blot detection of GFAP (upper panels). (**A**) Region 1, (**B**) Region 2, (**C**) Region 3, (**D**) Region 4, and (**E**) Region 5 as described in Figure 3. Chemiluminescence signals for GFAP were digitally captured and shown in the upper panels. GAPDH was also detected from the same blots and used as a reference for loading variability (lower panels). The captured GFAP signals were quantified using LI-COR Image Studio Lite software and normalized to GAPDH signals. Values shown in the bar graphs indicate the group average ± SEM. $^{**}p < 0.01$ (unpaired t-test), $n = 8$.

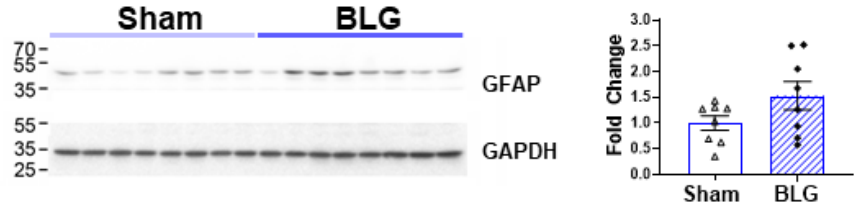
A. Region 1: Prefrontal & frontal cortices/striatum



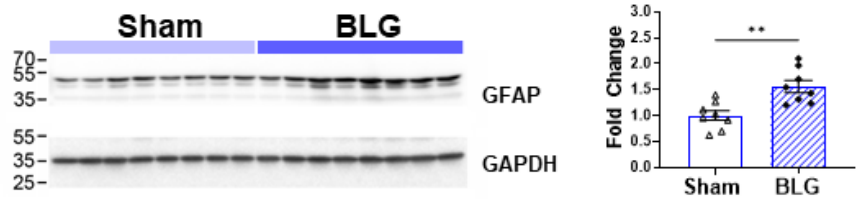
B. Region 2: Parietotemporal cortices/hippocampus



C. Region 3: Thalamus/hypothalamus



D. Region 4: Midbrain/rostral brainstem



E. Region 5: Cerebellum

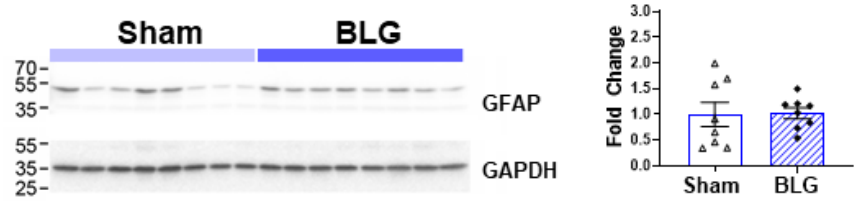
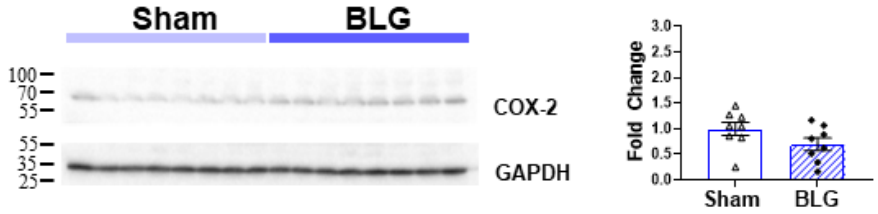
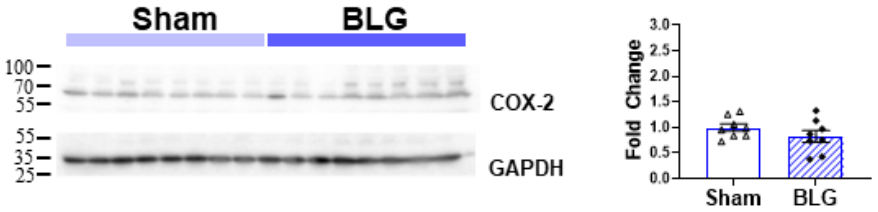


Figure 9. Western blot analysis of COX-2 in the isolated five brain regions. Soluble proteins isolated from the five regions were resolved on discontinuous 15% SDS-polyacrylamide gels for western blot detection of COX-2 (upper panels). (**A**) Region 1, (**B**) Region 2, (**C**) Region 3, (**D**) Region 4, and (**E**) Region 5 as described in Figure 3. Chemiluminescence signals for COX-2 were digitally captured and shown in the upper panels. GAPDH was also detected from the same blots and used as a reference for loading variability (lower panels). The captured COX-2 signals were quantified using LI-COR Image Studio Lite software and normalized to GAPDH signals. Values shown in the bar graphs indicate the group average ± SEM. $^{**}p < 0.001$ (unpaired t-test), $n = 8$.

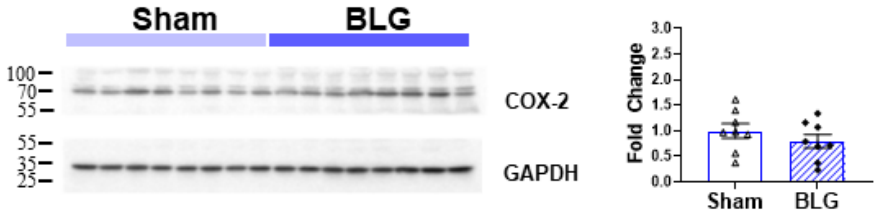
A. Region 1: Prefrontal & frontal cortices/striatum



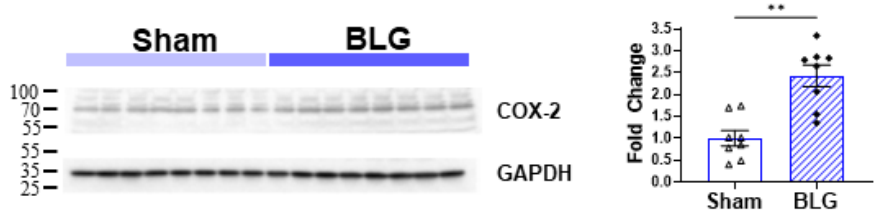
B. Region 2: Parietotemporal cortices/hippocampus



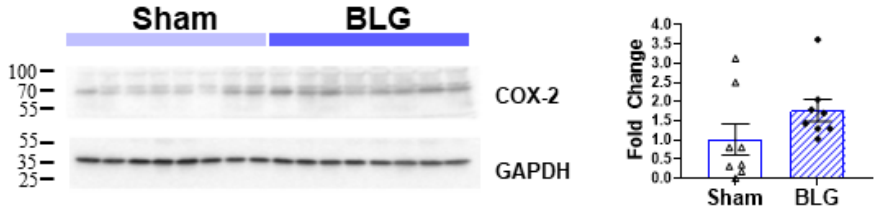
C. Region 3: Thalamus/hypothalamus



D. Region 4: Midbrain/rostral brainstem



E. Region 5: Cerebellum



The Proinflammatory Cytokine, TNFα, Was Elevated in the Midbrain Region

Based on our observation of astrogliosis and elevated COX-2 protein levels in the midbrain regions, we hypothesized that the GFAP-positive reactive astrocytes might be responding to and/or producing inflammatory mediator(s). Since astrocytes are capable of producing and responding to TNFα (Eddleston and Mucke, 1993), we measured the levels of this proinflammatory cytokine in the midbrain region (Region 4) using ELISA (Figure 10). As predicted, the amount of TNFα was significantly elevated in this region of BLG-sensitized mice by ~2.7-fold (sham: 1,273 ± 384 pg/mL; BLG: 3,469 ± 194 pg/mL, n = 8). This result demonstrated that proinflammatory events are present at least in this region of the brain of BLG-sensitized mice and provided the evidence that sensitization to a milk allergen results in neuroinflammation associated with behavioral abnormality.

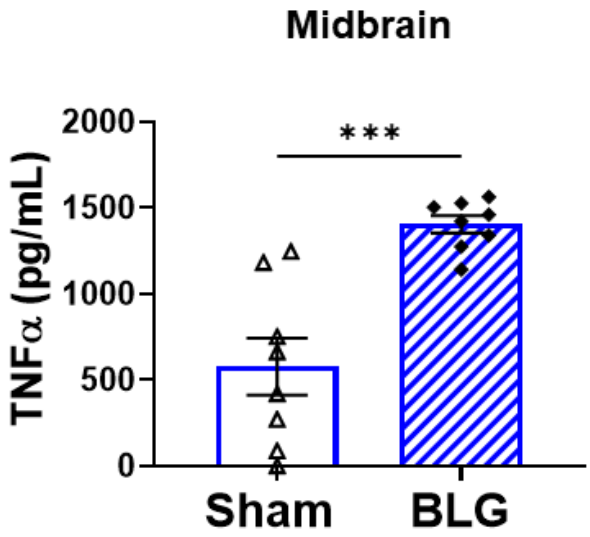


Figure 10. Quantification of TNFα levels in the midbrain region of sham and BLG male mice using ELISA. The levels of TNFα in the midbrain region were quantified by ELISA. Values indicate the group average ± SEM. ***$p <$ 0.001 (unpaired t-test), $n = 8$.

CHAPTER IV

RESULTS

Study 2 – Differential Myelination and Blood-Brain Barrier Associated Pathway Activation in Non-anaphylactic Model of Cow's Milk Allergy

Introduction

Food allergy is defined as a reproducible immune response to a specific food protein or allergen (Boyce et al., 2010). Classically food allergy is defined by an immediate IgE-dependent mechanism causing systemic anaphylaxis, respiratory distress, swelling, and hives (du Toit et al., 2010; Burks et al., 2012; Mousan and Kamat, 2016). However, for many years it has been shown that food allergies are comorbid with various behavioral disorders. Children with food allergies are more likely to be diagnosed with ADHD, anxiety, depression, and autism (Tryphonas and Trites, 1979; Patten and Williams, 2007; Yaghmaie et al., 2013; Garg and Silverberg, 2014; Shanahan et al., 2014; Zerbo et al., 2015; Ferro et al., 2016; Topal et al., 2016). Despite the behavioral effects of food allergy being well characterized, an exact mechanism has yet to be elucidated.

One reason for the gap in understanding is likely due to the inherent variability in food allergies and symptom presentation (Hill and Hosking, 1995; Baehler et al., 1996; Rona et al., 2007; Dupont, 2014). For example, the allergic response can be broken up into the immediate IgE-dependent mast cell response and the late phase response.

The late phase can last hours or days after the immediate phase is cleared and is underresearched (du Toit et al., 2010; Mousan and Kamat, 2016). For mast cells specifically, histamine release is commonly the primary focus of intervention; however, mast cells broadly and selectively release factors other than histamine, such as proteases, leukotrienes, and cytokines (Marshall, 2004; da Silva et al., 2014). The cytokine response is well known to amplify the proinflammatory reaction by stimulating many cell types and drive specific actions within the tissue. There is abundant evidence of CNS-directed responses in both cytokines and mast cells potentially acting as the initiators of neuroinflammation and causing behavioral changes (Bradding, 1999; Zorrilla et al., 2001; Hofmann and Abraham, 2009; Li et al., 2009; Nautiyal et al., 2012; Abbott et al., 2015).

The risks of behavioral changes are not unique to overt allergic reactions, however. An additional point of variability in food allergies is that some patients are non-anaphylactic and experience subclinical symptoms. These patients are less likely to be diagnosed or avoid their allergens, putting them at increased risk of repeated exposure. Combined, these factors highlight a subpopulation of patients who are sub-anaphylactic and are of increased risk for neuropsychiatric diagnosis. Therefore, due to their behavioral symptoms' atypical etiology, such patients are likely resistant to typical neuropharmacological intervention (Al-Harbi, 2012; Hirschtritt et al., 2017). To identify and treat these patients, it is necessary to improve diagnostic criteria and establish a mechanism by which peripheral allergy-induced inflammation can influence behavior.

To elucidate a potential mechanism, we sensitized C57BL/6J mice to the cow's milk allergen β-lactoglobulin. We then compared sensitized mice to sham mice to characterize a clinical phenotype and neuropathology using RNA-sequencing and

immunohistochemistry. We report that transcriptional changes were found within the brain in a region-specific manner. These transcriptional changes were observed in the midbrain region previously identified as being impacted by food allergy (Germundson et al., 2018; Smith et al., 2019). The transcriptome changes implied increased blood vessel permeability and altered glial cell function within the midbrain. Increased blood vessel permeability caused by the blood-brain barrier's breakdown was further validated in BLG-sensitized mice using immunohistochemistry for serum proteins. These findings support disruption of normal barrier function being a critical step in the peripheral to central signaling mechanism for food allergy-induced psychiatric symptoms.

Lack of Anaphylactic Symptoms in BLG-Sensitization of C57BL/6J Mice Despite Increased Serum Allergen-specific IgE

A weekly recording of body weight was used to monitor the health of two groups of mice. Starting one week prior to the first week of sensitization until the week of sacrifice, the weights were recorded the day before administering vehicle or BLG containing solutions (Figure 1B, C). Mice set aside for transcriptomic analysis were sacrificed following challenge (Figure 1B), while mice set aside for anaphylactic scoring and histological analysis were sacrificed following testing (Figure 1C) (Week 6). Analysis of weight showed no difference in health between sham and BLG-treated mice based on comparable growth (Figure 11A).

During the week 6 challenge, 30 min after gavage, observable anaphylactic symptoms were scored, and internal body temperature was recorded (Figure 11A and 11B, respectively). No difference in apparent anaphylaxis symptoms were observed in either group of mice, though 1 BLG mouse was observed to have reduced activity following

challenge (Figure 11B). Likewise, there were no significant temperature deviations present in either group of mice following challenge (Figure 11C). These data demonstrate a lack of physical allergic response following sensitization and allergen re-exposure.

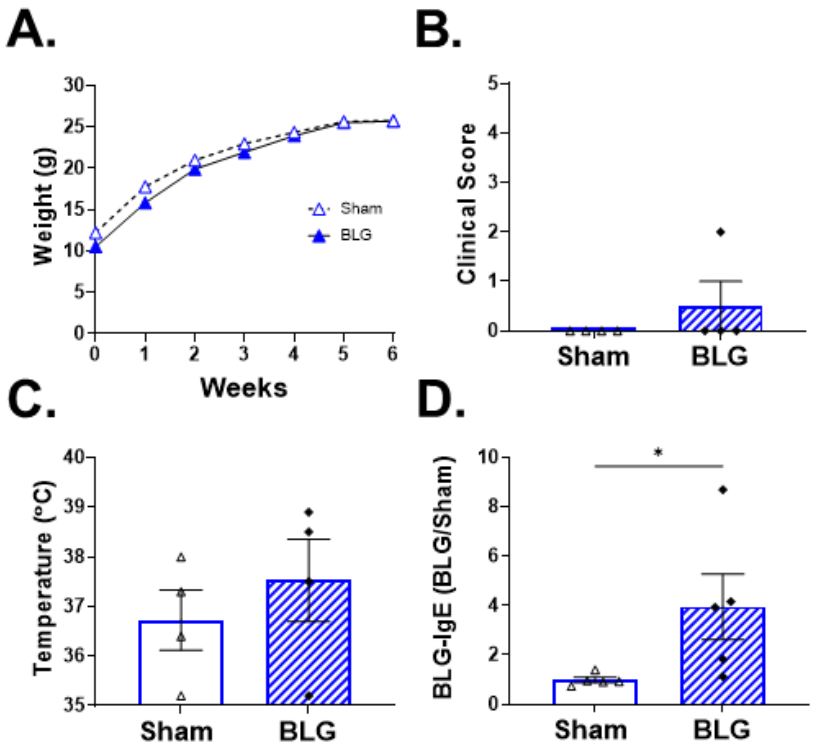


Figure 11. Clinical assessment of health and sensitivity of mice. (**A**) Body weight growth curve shows comparable weight gain throughout the process of sensitization between sham (open symbols) and BLG-sensitized (filled symbols) mice. (**B**) Symptoms were scored based on the symptom score table (Table 1). (**C**) Body temperature (°C) was measured 30 min after the allergen challenge. (**D**) BLG-specific IgE detected from terminal serum using ELISA. Fold change was calculated by normalizing optical density (OD) values obtained for BLG-sensitized (striped bars with filled diamonds) groups to sham groups (open bars with open triangles). Values shown in the graphs indicate the group average ± SEM, *$p < 0.05$ (Mann-Whitney test), n = 4-5.

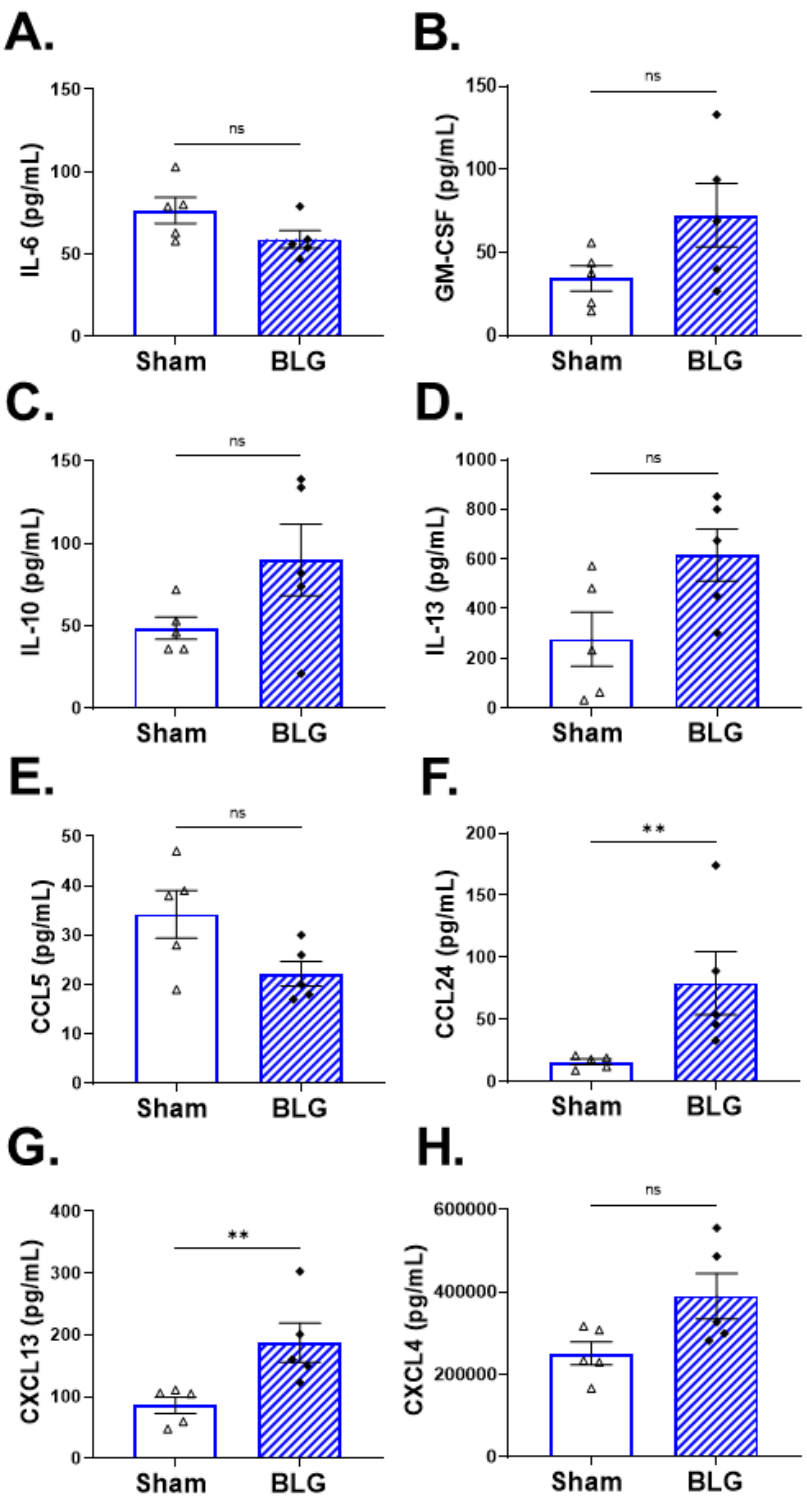
To ensure BLG-sensitivity was achieved, BLG-specific IgE was detected using ELISA (Figure 11D). Serum BLG-specific IgE was found to be elevated in sensitized mice despite a lack of anaphylactic symptoms. BLG-sensitized mice had increased fold-OD compared to untreated sham mice (sham: 0.93 ± 0.11; BLG 3.95 ± 1.32; $n = 5$ in all groups, $p < 0.05$, Mann-Whitney test). Results indicate that BLG hypersensitivity was established in BLG-sensitized mice following the treatment regime.

Plasma CCL24 and CXCL13 Significantly Increased Accompanied by Trended Increases in Other Th2 Cytokines

Knowing that sensitization was achieved despite no apparent allergic symptoms, collected plasma was used to quantify circulating cytokines. Using multiplex ELISA, a panel of cytokines, chemokines, and associated immunological factors were quantified. BLG-sensitized mice had 2 chemokines significantly increased over sham mice (Figure 12). CCL24 (C-C motif chemokine ligand 24/eotaxin-2) a chemokine for eosinophils was increased approximately 5-fold in BLG mice (Figure 12F; Sham: 15.8 ± 2.3 pg/mL; BLG: 79.2 ± 25.5 pg/mL; $n = 5$ in all groups, $p < 0.01$, Mann-Whitney test). CXCL13 (C-X-C motif chemokine ligand/B-lymphocyte chemoattractant) a chemokine for T and B-lymphocyte was increased 2.1-fold in BLG mice (Figure 12G; Sham: 86.0 ± 13.2 pg/mL; BLG: 187.4 ± 31.5 pg/mL; $n = 5$ in all groups, $p < 0.01$, Mann-Whitney test). Though not significant, a trend for increase in IL-10 (Figure 12C) and IL-13 (Figure 12D) was observed in BLG mice as previously reported in C57BL/6J mice (Smith et al., 2021). In addition to these previously identified factors, CXCL4 (Figure 12H) and GM-CSF (Figure 12B) were also slightly increased in BLG mice, though not statistically significant. These analytes are suggestive of a Th2-type response

elevated in BLG-sensitized C57BL/6J mice. Many increased factors are routinely found elevated in allergic individuals or are central to the establishment of IgE-mediated hypersensitivity. These data provide further evidence that sensitization was achieved and suggest a role these analytes play in the systemic effects observed in our model system.

Figure 12. Levels of immune mediators included in the Quantibody Mouse Cytokine Array 5 (QAM-CYT-5) were quantified from plasma samples. The abundances of (**A**) IL-6, (**B**) GM-CSF, (**C**) IL-10, (**D**) IL-13, (**E**) CCL5, (**F**) CCL24, (**G**) CXCL13, and (**H**) CXCL4 were quantified. Sham mice (open bars with open triangles); BLG mice (striped bars with filled diamonds). Bars indicate group average values ± SEM $^{**}p < 0.01$ (Mann-Whitney test), $n = 5$.


A.
IL-6 (pg/mL)
150
100
50
0
ns
Sham
BLG
B.
GM-CSF (pg/mL)
150
100
50
0
ns
Sham
BLG
C.
IL-10 (pg/mL)
150
100
50
0
ns
Sham
BLG
D.
IL-13 (pg/mL)
1000
800
600
400
200
0
ns
Sham
BLG
E.
CCL5 (pg/mL)
50
40
30
20
10
0
ns
Sham
BLG
F.
CCL24 (pg/mL)
200
150
100
50
0
**
Sham
BLG
G.
CXCL13 (pg/mL)
400
300
200
100
0
**
Sham
BLG
H.
CXCL4 (pg/mL)
600000
400000
200000
0
ns
Sham
BLG

Impact of BLG-Sensitization on Gut Health and Intestinal Permeability

Previous results have eluded to changes in gut health resulting from non-anaphylactic BLG allergy (Smith et al., 2019). RNA was extracted from the ileum, and the transcripts of proinflammatory cytokines *Tnfα* and *IL-1β*, Th2 promoting factor *Il-4*, and tight junction protein *Ocln* were quantified via RT-qPCR. The tested factors varied greatly within groups, likely causing none of the factors being significantly different. *Il-1β* was increased approximately 1.5-fold in BLG-sensitized mice, while *Ocln* was reduced to around 0.5-fold in BLG mice (Figure 13). Though not conclusive, these data coupled with previously identified occludin protein reduction (Smith et al., 2019) justified quantifying gut permeability.

Mice were given FITC-dextran, and the amount that crossed the gut barrier was quantified from the serum. The exact amount of FITC fluorescence varied within groups (Figure 14). There were no differences between groups, both sham and BLG mice having 0.24-0.26 μg/mL FITC-dextran in their serum. Based on these data, there appears to be no evidence of gut pathology or permeability resulting from sensitization.

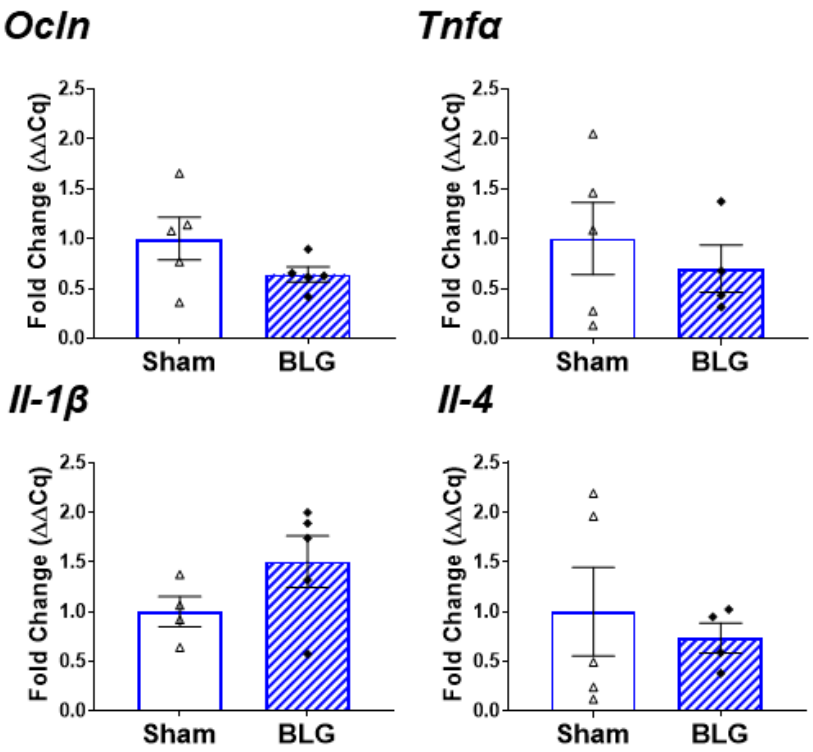


Figure 13. Ileum RT-qPCR fold change in transcription. Transcripts for *Ocln*, *Tnfα*, *Il-1β*, and *Il-4* were quantified from ileum extracted RNA. Sham mice (open bars with open triangles); BLG mice (striped bars with filled diamonds). Bars indicate Cq values for *Ocln* and *Tnfα* were normalized to the Cq values of Gapdh (ΔCq) to calculate the expression values ($\Delta Cq = 2^{-\Delta Cq}$). Values shown in the bar graphs are expressed as the fold change (ΔΔCq) ± SEM (Mann-Whitney test), $n = 5$.

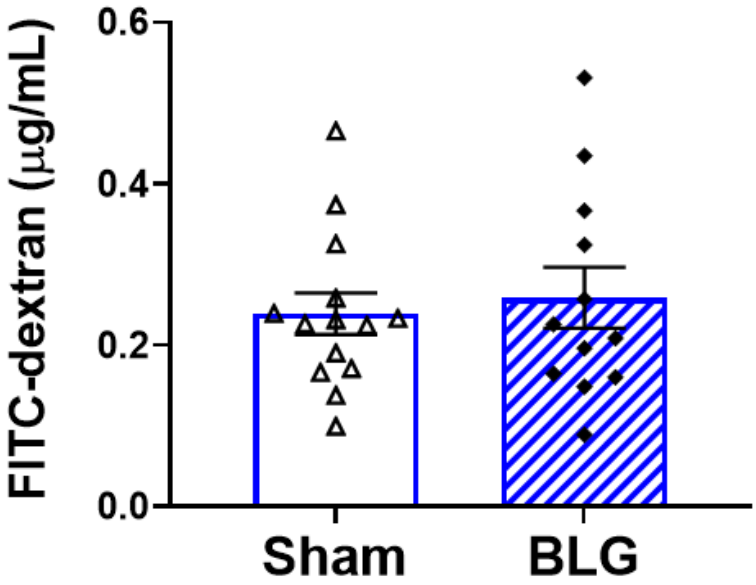


Figure 14. Serum FITC-dextran concentration. Mice were given 4 kDa FITC-dextran to assess the amount crossing the gut barrier. FITC fluorescence at an emission of 528 nm was recorded to detect abundance in serum. Sham mice (open bars with open triangles); BLG mice (striped bars with filled diamonds). Bars indicate group average values ± SEM (Mann-Whitney test), n = 12-14.

Regional Brain Transcriptional Changes Resulting from BLG-Sensitization

Brains extracted from mice set aside for transcriptomic analysis were divided regionally (Figure 3). RNA was extracted from region 1 (STR) containing the striatum and frontal cortex; region 2 (HPC) containing the hippocampus, temporal and parietal cortices; region 3 (THAL) containing the thalamus and hypothalamus; and region 4 (MB), which contained the midbrain, and were sequenced. Fold change values of differentially expressed genes generated from RNA sequencing were used as input values for IPA. Upstream regulator activation states of regional transcriptome profiles were identified. A small set of regulators common across brain regions was identified from this analysis (Figure 15). Dio2 was found to have a negative z-score of activation in STR (z = -1.067), HPC (z = -2.360), and MB (z = -1.342) regions. Slc16a2, similarly, had negative z-scores across the STR (z = -1.000), HPC (z = -1.633), and MB (z = -0.577) regions. Psen1 had a negative z-score of activation in the STR (z = -0.269) and HPC (z = -0.269), while Eomes was positive in the STR (z = 1.000) and HPC (z = 1.000). Fmr1 was suppressed in the STR (z = -1.342) but active in the MB (z = 1.383). Notable Sox2 signaling was also found highly suppressed in the MB region (z = -2.538).

With upstream regulators identified based upon the transcriptome profiles, we then organized regionally differentially expressed genes into canonical pathways and calculated an activation z-score (Figure 16). Within the STR a broad spectrum of pathways were differentially activated or suppressed. Notably, endocannabinoid developing neuron pathways (z = -1.414), endometrial cancer signaling (z = -1.000), IL-7 signaling (z = -1.000), and B cell receptor signaling pathways (z = -0.707) were found to

have negative activation z-scores, while eNOS (z = 1.890), NFAT (z = 1.732), white adipose tissue browning (z = 1.732), Gαs (z = 1.414), nitric oxide (z = 1.414), synaptogenesis (z = 1.414), cAMP (z = 1.213), opioid (z = 0.943), Fcγ receptor mediated phagocytosis signaling pathways (z = 0.816) were activated. In the THAL the white adipose tissue browning pathway (z = -0.707) had a negative activation z-score while netrin (z = 1.890) and Th17 signaling (z = 2.000) was activated. However, in the MB FcεRI (z = 0.816) and amyotrophic lateral sclerosis signaling (z = 1.000) were selectively activated.

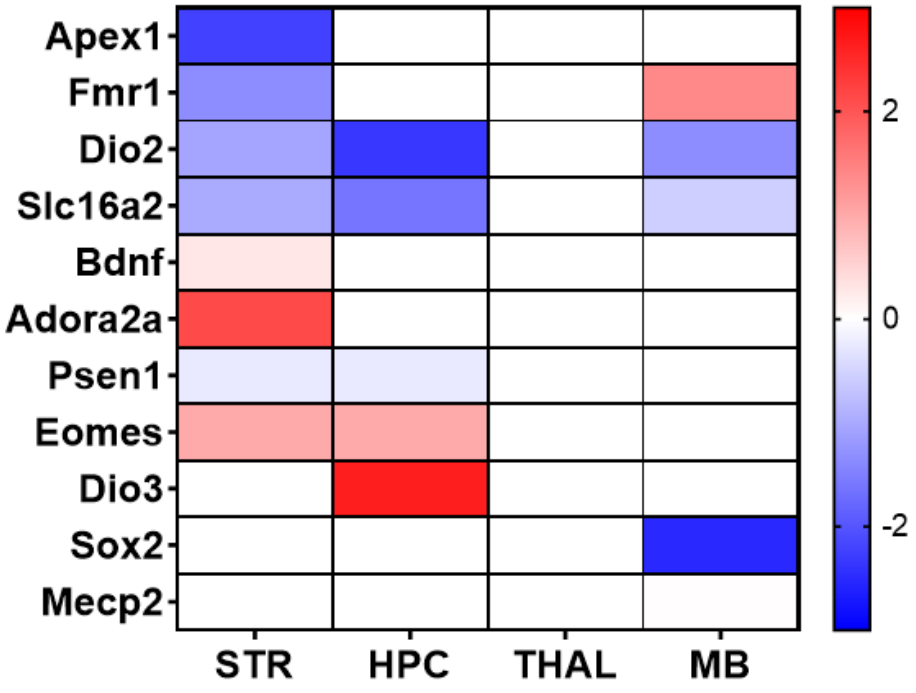


Figure 15. Ingenuity pathway analysis of regional upstream transcriptional regulators. A subset of statistically significant differentially activated upstream regulators were identified across multiple brain regions, including STR, HPC, THAL, MB (Figure 3). Heatmap color corresponds to activation z-score (BLG/Sham), *n* = 3.

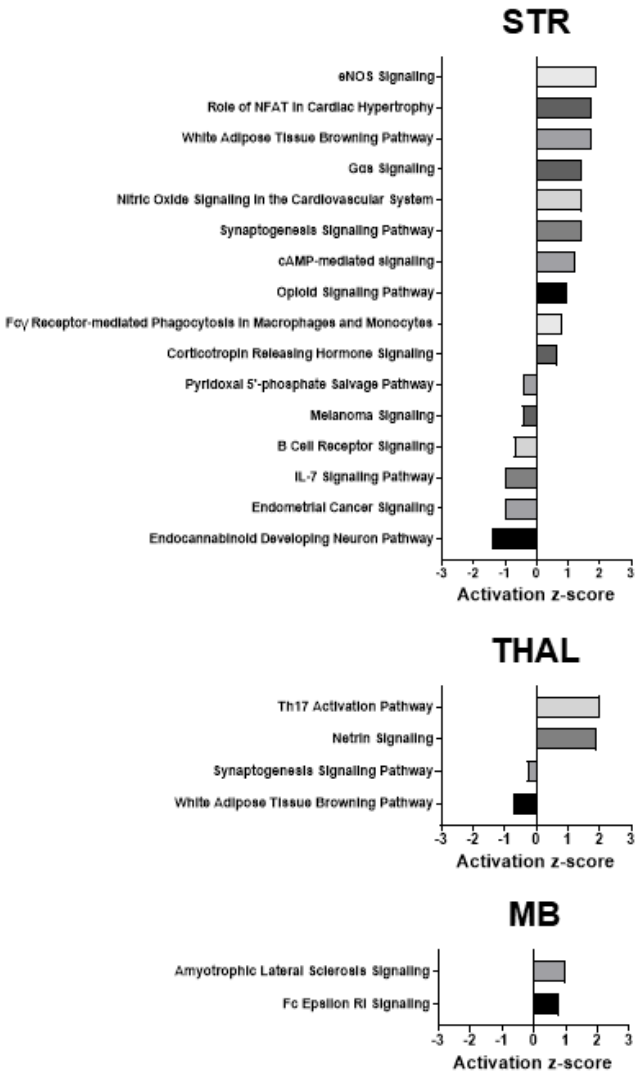
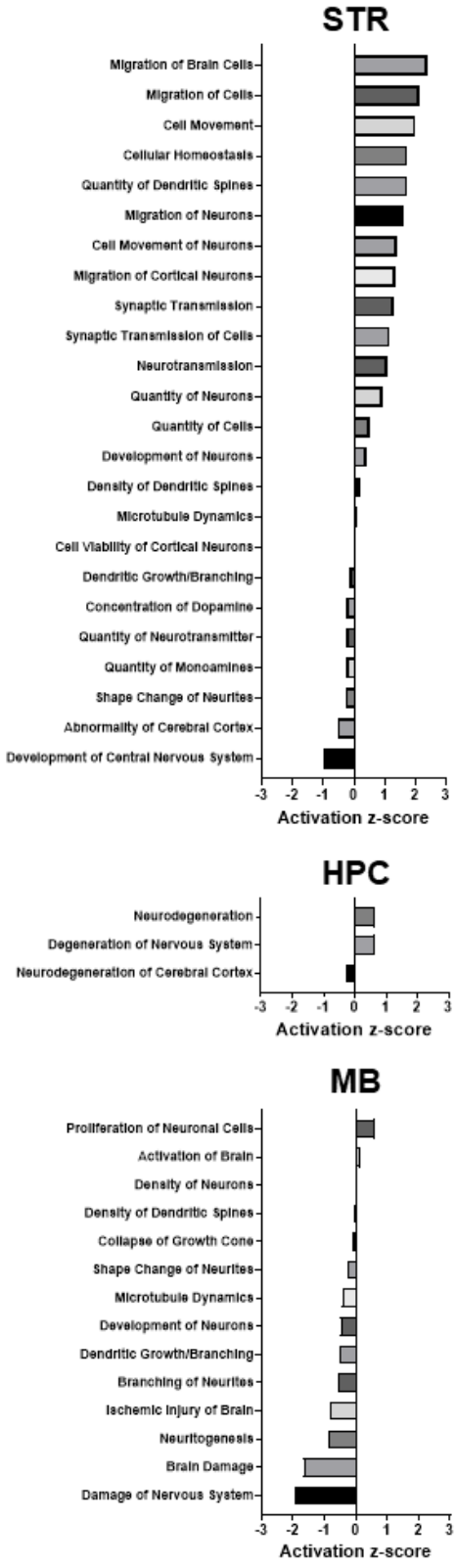


Figure 16. Ingenuity pathway analysis regional canonical pathway activation. Regional transcriptomes were analyzed in the IPA platform, and statistically significant differentially expressed genes (Based on *p*-value) were placed into canonical pathways based on database information. Log_2 fold change values (BLG/sham) were used as input values. From the number of differentially expressed genes within a pathway, the activation z-score was calculated. All displayed pathways were statistically significant $*p < 0.05$, $n = 3$.

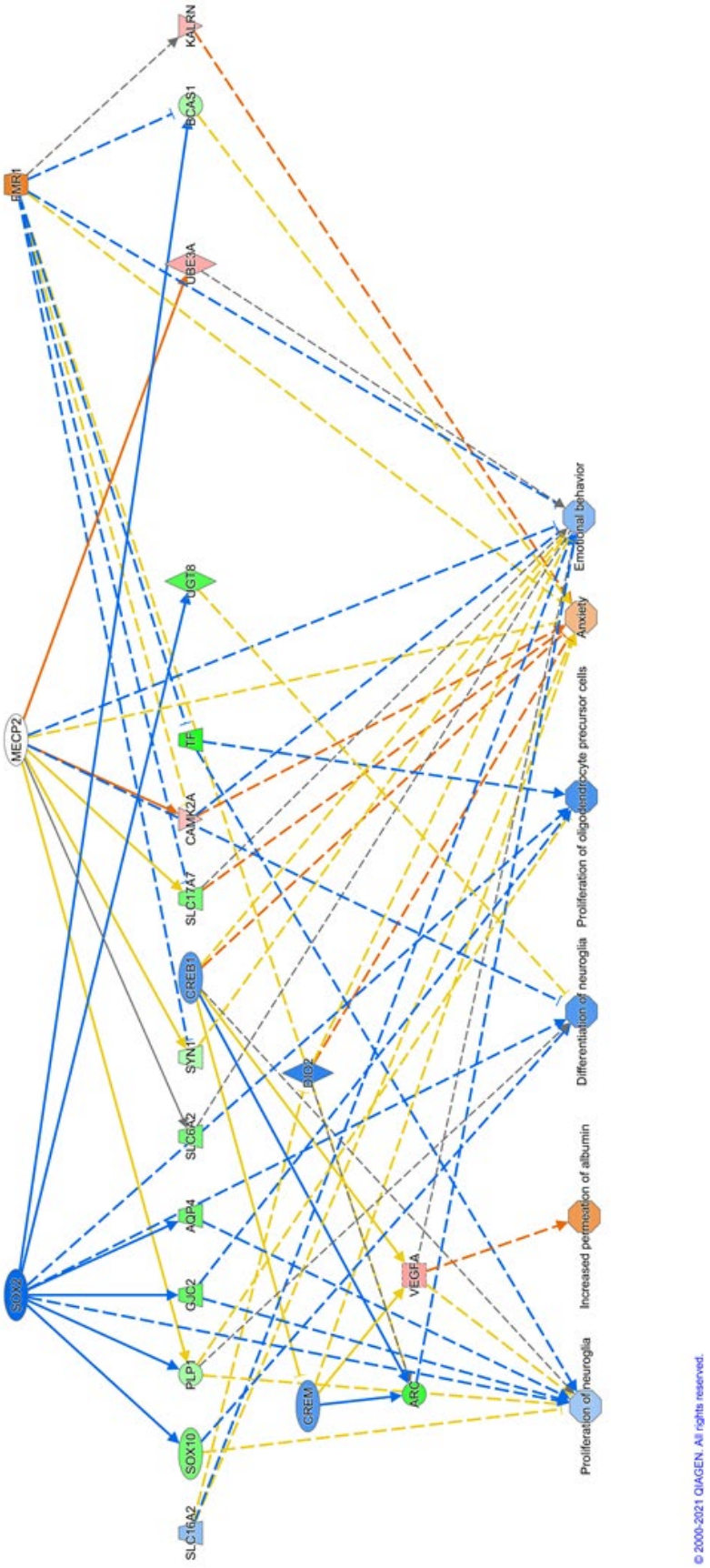
We further performed disease state and cell function pathway analysis to assess the changes in cellular behavior and health of the brain resulting from peripheral allergy (Figure 17). In the STR, pathways involved in cell migration of brain cells/neurons ($z = 2.400$), the quantity of dendritic spines ($z = 1.732$), synaptic transmission ($z = 1.166$), and the quantity of neurons ($z = 0.928$) were activated, while central nervous system development pathway ($z = -0.970$) was inactivated. Meanwhile, in the MB, inactivation of pathways associated with damage of the nervous system ($z = -1.938$), neuritogenesis ($z = -0.865$), and ischemic injury of the brain ($z = -0.818$) were observed.

Figure 17. Ingenuity pathway analysis regional disease state pathway activity. Regional transcriptomes were analyzed in the IPA platform, and statistically significant differentially expressed genes (Based on *p*-value) were placed aligned with disease state and cell function pathways based on database information. Log_2 fold change values (BLG/sham) were used as input values. From the number of differentially expressed genes within a pathway, the activation z-score was calculated. All displayed pathways were statistically significant $*p < 0.05$, $n = 3$


STR
Migration of Brain Cells
Migration of Cells
Cell Movement
Cellular Homeostasis
Quantity of Dendritic Spines
Migration of Neurons
Cell Movement of Neurons
Migration of Cortical Neurons
Synaptic Transmission
Synaptic Transmission of Cells
Neurotransmission
Quantity of Neurons
Quantity of Cells
Development of Neurons
Density of Dendritic Spines
Microtubule Dynamics
Cell Viability of Cortical Neurons
Dendritic Growth/Branching
Concentration of Dopamine
Quantity of Neurotransmitter
Quantity of Monoamines
Shape Change of Neurites
Abnormality of Cerebral Cortex
Development of Central Nervous System
-3 -2 -1 0 1 2 3
Activation z-score
HPC
Neurodegeneration
Degeneration of Nervous System
Neurodegeneration of Cerebral Cortex
-3 -2 -1 0 1 2 3
Activation z-score
MB
Proliferation of Neuronal Cells
Activation of Brain
Density of Neurons
Density of Dendritic Spines
Collapse of Growth Cone
Shape Change of Neurites
Microtubule Dynamics
Development of Neurons
Dendritic Growth/Branching
Branching of Neurites
Ischemic Injury of Brain
Neuritogenesis
Brain Damage
Damage of Nervous System
-3 -2 -1 0 1 2 3
Activation z-score

Based upon previously observed differences in the MB, we focused on MB upstream regulators, canonical pathways, and anxiety behavior. A predictive pathway was constructed in IPA to investigate potential relationships between identified differentially expressed genes, predicted upstream regulators, disease states and canonical pathways of interest. Differentially expressed genes and the pathways for the proliferation and differentiation of glial cells, albumin permeability, the proliferation of oligodendrocyte precursors, anxiety, and emotional behavior were placed into a causal network. Based on the quantified differentially expressed genes and predicted activity states of the upstream regulators, these pathways' effects were predicted (Figure 18). The network predicted that increased permeability of albumin and anxiety behavior would occur, while glial/oligodendrocytes proliferation and differentiation and emotional behavior are decreased. These data are suggestive that glial cell function such as oligodendrocytes and astrocytes were inhibited, and capillaries are permeable to serum proteins compromising the blood-brain barrier.

Figure 18. Midbrain causal network analysis. Upstream regulators within the midbrain and differentially expressed genes downstream of those regulators were placed into a network with using Qiagen IPA software based upon established connections within the database. The factors were placed in the network, and IPA generated an activation state for the pathways to evaluate the connection between differentially activated regulators, differentially expressed genes, and pathways of interest.


KALRN
BCAS1
EMR1
UBE3A
UGT8
MECP2
ITF
CAMK2A
SLC17A7
GREB1
SYN1
PI0X
SLC6A2
AQP4
GJC2
VEGFA
PLP1
CREM
ARC
SOX10
SLC16A2
SOX2
Emotional behavior
Anxiety
Proliferation of oligodendrocyte precursor cells
Differentiation of neuroglia
Increased permeation of albumin
Proliferation of neuroglia
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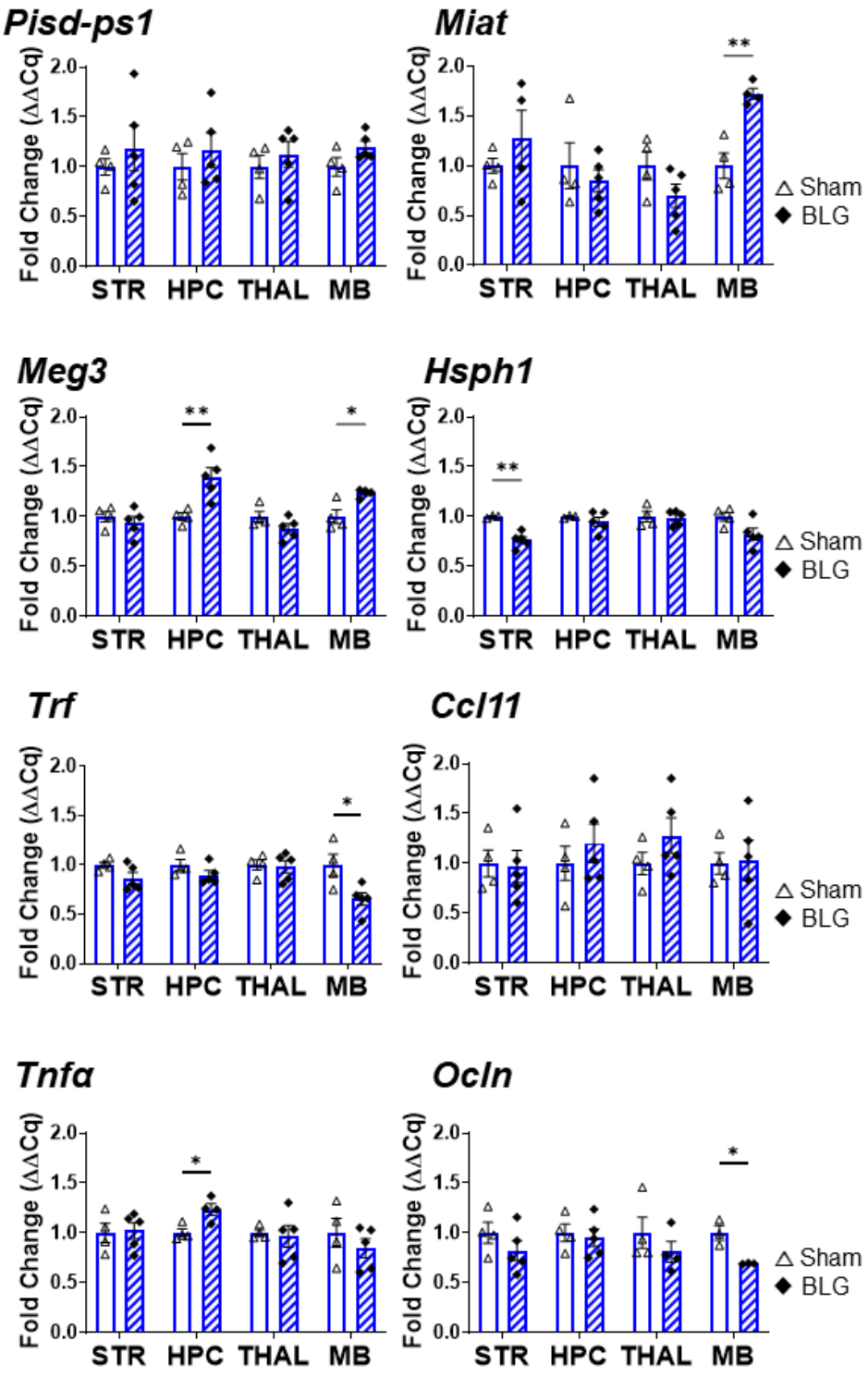
To validate our sequencing results and factors central to the proposed mechanism between BLG-sensitization and behavioral changes. RT-qPCR was performed from the 4 brain regions for *Pisd-ps1*, *Miat*, *Meg3*, *Hsph1*, *Trf*, *Ccl11*, *Tnfα*, and *Ocln* (Figure 19). The STR had reduced transcription on Hsph1 in BLG-sensitized mice (Sham: 1.000 ± 0.008, $n = 3$; BLG: 0.765 ± 0.034, $n = 5$; $p < 0.01$, t-test). Within the HPC region increased *Meg3* (Sham: 1.000 ± 0.052, $n = 4$; BLG: 1.399 ± 0.093, $n = 5$; $p < 0.01$, t-test) and *Tnfα* (Sham: 1.000 ± 0.098, $n = 4$; BLG: 1.235 ± 0.057, $n = 4$; $p < 0.05$, t-test). No differences were observed in the THAL region for the validated genes, however in the MB, *Miat* (Sham: 1.000 ± 0.076, $n = 4$; BLG: 1.274 ± 0.281, $n = 4$; $p < 0.01$, t-test), *Meg3* (Sham: 1.000 ± 0.070, $n = 4$; BLG: 1.236 ± 0.021, $n = 4$; $p < 0.05$, t-test), *Trf* (Sham: 1.000 ± 0.111, $n = 4$; BLG: 0.656 ± 0.034, $n = 5$; $p < 0.05$, t-test), and *Ocln* (Sham: 1.000 ± 0.074, $n = 3$; BLG: 0.692 ± 0.003, $n = 3$; $p < 0.05$, t-test) were differentially expressed. From these data it is clear that there is evidence for BLG-mediated blood vessel permeability and disrupted iron transport resulting from decreased *Ocln* and *Trf*, respectively.

Figure 19. Validation of differentially expressed genes using RT-qPCR. The fold change of *Pisd-ps1*, *Miat*, *Meg3*, *Hsph1*, *Trf*, *Ccl11*, *Tnf*α, and *Ocln* were evaluated to validate RNAseq findings. Sham mice (open bars with open triangles); BLG mice (striped bars with filled diamonds). Bars indicate group The Cq values for *Ocln* and *Tnfα* were normalized to the Cq values of *Gapdh* (ΔCq) to calculate the expression values ($\Delta Cq = 2^{-\Delta Cq}$). Values shown in the bar graphs are expressed as the fold change (ΔΔCq) ± SEM (t-test) $*p < 0.05$, $**p < 0.01$, $n = 3\text{-}5$.


Pisd-ps1
Miat
Meg3
Hsph1
Trf
Ccl11
Tnfα
Ocln
Fold Change (ΔΔCq)
2.0
1.5
1.0
0.5
0.0
STR
HPC
THAL
MB
**
*
△ Sham
◆ BLG

Increased Capillary IgG Permeability in Midbrain of BLG-Sensitized Mice

Based upon our findings at the RNA level, along with pathway analysis and previously observed astrogliosis (Smith et al., 2019), we focused on the immunoreactivity of myelin and blood-brain barrier integrity. The disruption of *Trf* and its role in oligodendrocyte development and function had great potential for impacting myelination in the brain. Myelination was investigated by staining for MBP. Despite the convincing loss of *Trf* transcription and pathway evidence for disruption of oligodendrocyte function, no evidence of differential MBP staining was observed (Figure 20). From these data, we found no evidence of differential MBP optical density between sham and BLG-treated mice.

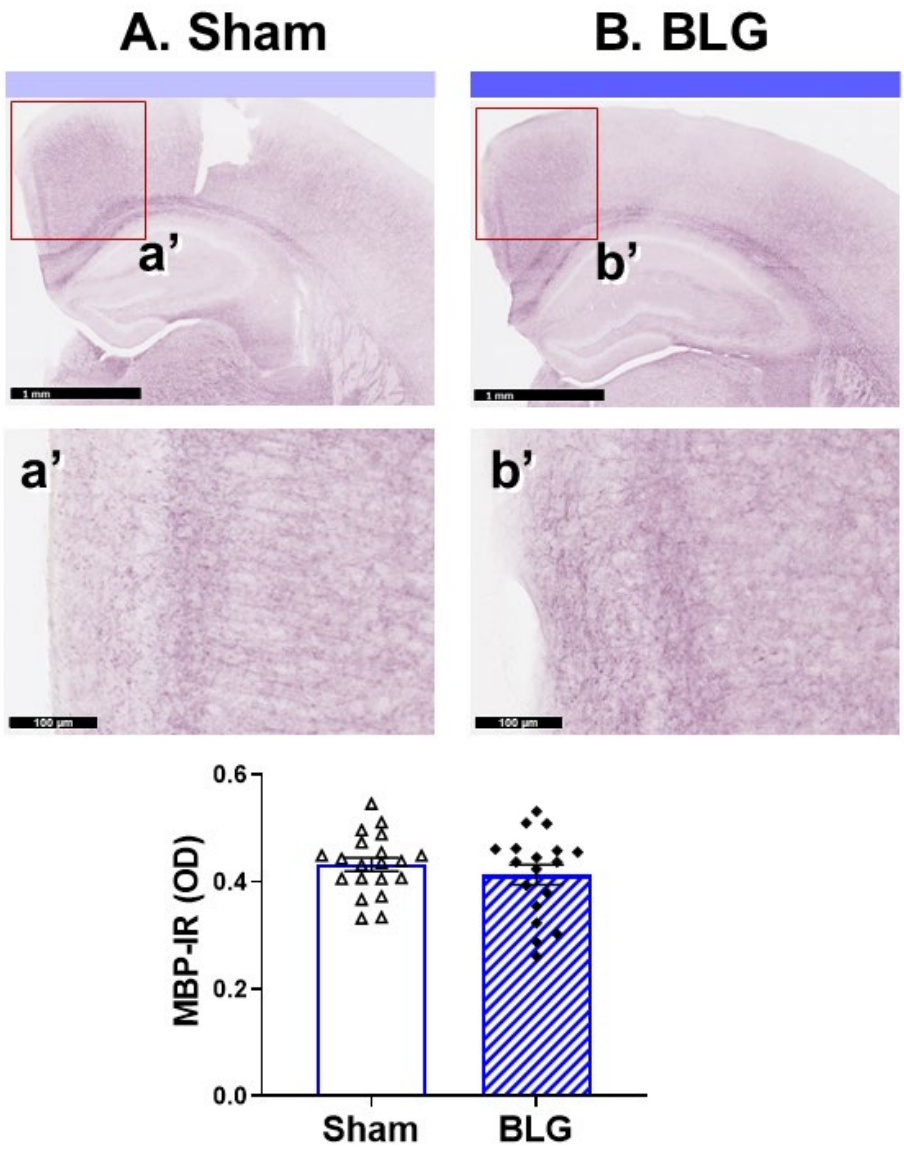


Figure 20. Immunoreactivity of MBP in retrosplenial cortex of sham and BLG sensitized mice. MBP-positive myelin and oligodendrocytes were identified by immunohistochemically staining brain sections (40 µm). Representative cortex sections from sham (**A**, **a'**) and BLG-sensitized (**B**, **b'**) male mice are shown. The red rectangles in (**A**, **B**) indicate where the respective higher magnification images a' and b' were taken. The low-magnification (**A**, **B**) and high-magnification (**a'-b"**) images taken with a 2.5X and 20X objectives, respectively. Scale bars: 1 mm for (**A**) and (**B**); 100 µm for **a'** and **b'**. Quantification of optical density was performed using QuPath. Sham mice (open bars with open triangles); BLG mice (striped bars with filled diamonds); bars indicate group average values ± SEM (Mann-Whitney test), n = 18-20.

The reduction of *Ocln* gene expression in the midbrain region suggests a breakdown in the tight junctions necessary for capillary structure and the selectively permeable barrier between the circulation and the brain parenchyma. To assess the integrity of the blood-brain barrier, we performed immunohistochemistry for the IgG. IgG is abundant in the circulation but is occluded from nervous tissue. In sham mice, well-defined intact capillaries were observed with light surrounding background (Figure 21a'), while in BLG mice, hazy light capillaries are observed with a much darker background (Figure 21b'). The differential staining between the groups can be observed throughout the midbrain; here, we represented sections of the substantia nigra pars reticulata (a'+b'). We then quantified the optical density of the IgG stain within the totality of representative sections. Optical density was higher in BLG-sensitized mice (Sham: 0.111 ± 0.012, $n = 7$; BLG: 0.192 ± 0.013, $n = 6$; $p < 0.001$, t-test). We futher investigated other brain regions (Supplemental Figure 2), including the cerebellum, an area where no differential staining was observed. In addition, we observed blood vessels in cross-section where high amounts of IgG was deposited in BLG-sensitized mice. These observations demonstrated increased blood vessel permeability, which has allowed for extravascular IgG to accumulate.

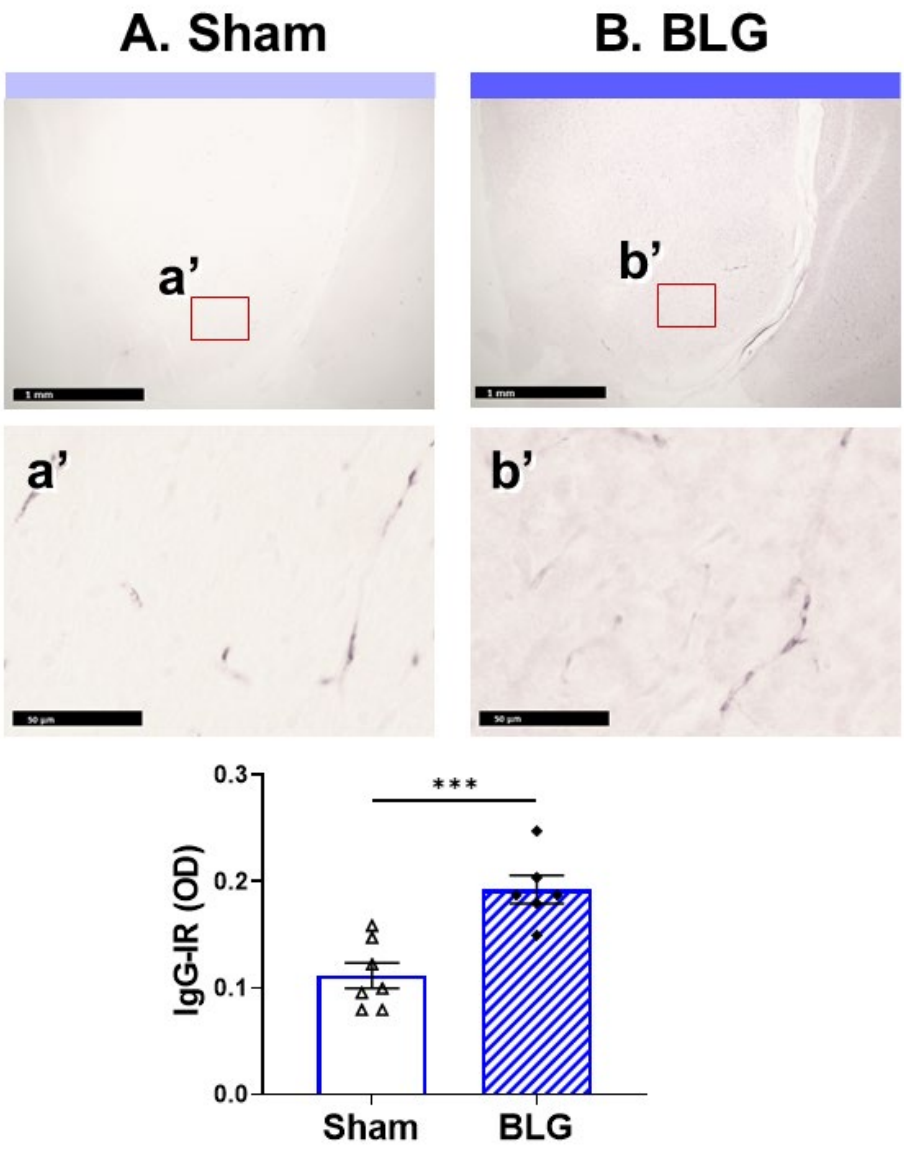


Figure 21. Immunoreactivity of IgG in midbrain region of sham and BLG-sensitized mice. The OD of IgG quantified extravascular IgG within the brain parenchyma (40 μm). Representative midbrain sections from sham (**A**, **a'**) and BLG-sensitized (**B**, **b'**) male mice are shown. The red rectangles in (**A**, **B**) indicate where the respective higher magnification images **a'-b"** were taken. The low-magnification (**A**, **B**) and high-magnification (**a'**, **b'**) images taken with a 2.5X and 40X objectives, respectively. Scale bars: 1 mm for (**A**) and (**B**); 50 μm for **a'** and **b'**. Quantification of optical density was performed using QuPath. Sham mice (open bars with open triangles); BLG mice (striped bars with filled diamonds); bars indicate group average values ± SEM (Mann-Whitney test), ***$p < 0.001$, n = 6-7.

CHAPTER V

RESULTS

Study 3 – Anxiety-like Behavior and Intestinal Microbiota Changes as Strain- and Sex-dependent Sequelae of Mild Food Allergy in Mouse Models of Cow's Milk Allergy

Contents of this chapter were originally published in Brain Behavior and Immunity (Smith et al., 2021)

Introduction

Food allergy, defined as "an adverse health effect arising from a specific immune response that occurs reproducibly on exposure to a given food" (Boyce et al., 2010), is an increasingly prevalent health concern worldwide (Mullins, 2007; Liu et al., 2010; Gupta et al., 2018; Loh and Tang, 2018; Sicherer and Sampson, 2018). In the United States, where approximately 8-10% of children and adults are afflicted with food allergy, cow's milk allergy (CMA) has been reported as the second most common food allergy in both age groups (Gupta et al., 2018; Gupta et al., 2019). Clinical presentations of CMA can vary across individuals, and their manifestations may be immediate or delayed (Hill and Hosking, 1995; Baehler et al., 1996; Koletzko et al., 2012; Dupont, 2014). Symptoms that are typically recognized as "allergic reactions" include edema, hives, diarrhea, vomiting, respiratory distress, and systemic anaphylaxis, which occur immediately after ingestion of milk via immunoglobulin E (IgE)-mediated responses (du Toit et al., 2010; Burks et al., 2012; Mousan and Kamat, 2016). Delayed symptoms of CMA are more generalized

cutaneous and gastrointestinal discomfort, such as eczema and constipation, and can emerge several hours to days following milk consumption via IgE-independent mechanisms (du Toit et al., 2010; Mousan and Kamat, 2016).

In addition to the clinical presentations mentioned above, mood, cognitive, and behavioral symptoms have been associated with CMA, and thus, neuropsychiatric disorders such as anxiety, depression, attention deficit hyperactivity disorder (ADHD), and autism may partly be neurological manifestations of hypersensitivity to cow's milk proteins and other dietary allergens in some individuals (Davison, 1949; Speer, 1954; Boris and Mandel, 1994; Hak et al., 2013; Lyall et al., 2015; Topal et al., 2016; Xu et al., 2018). In support of this notion, removal of suspected food from patients' diet has been reported to alleviate their symptoms while reintroduction exacerbates them (Davison, 1949; Speer, 1954; Boris and Mandel, 1994; Stevens et al., 2010). Furthermore, oral immunotherapy of children with CMA was found to significantly improve anxiety (Carraro et al., 2012). Despite a large number of case reports and cohort studies that demonstrated positive correlations between neuropsychiatric conditions and atopic diseases (Afari et al., 2001; Heaney et al., 2005; Mostafa et al., 2008; Yaghmaie et al., 2013; Garg and Silverberg, 2014; Lyall et al., 2015; Ferro et al., 2016; Goodwin et al., 2017; Busquets et al., 2019; Blöndal et al., 2020), CMA or other food allergies as a pathophysiological trigger of mood and behavioral symptoms has not been fully acknowledged in the field, but rather perceived as a psychological trigger of fear arising from the anticipation for accidental exposures to offending allergens (Cummings et al., 2010; Walkner et al., 2015; Herbert et al., 2016; Polloni and Muraro, 2020). Inconsistent results across clinical studies, perhaps due to multiple variables associated with the

cohorts, have also likely contributed as inconclusive evidence for the role of food allergy as a causal factor for neuropsychiatric symptoms. Indeed, variables, such as genetic background and ethnicity, diet, and medical history, are challenging to normalize with human subjects in addition to the differences in the presentations, number, and severity of food allergies. Furthermore, intestinal microbiota, which has been increasingly implicated in both allergy (Inoue et al., 2017; Kourosh et al., 2018; Hussain et al., 2019) and neuropsychiatric disorders (Finegold et al., 2002; Wang et al., 2013; Naseribafrouei et al., 2014; Kelly et al., 2016; Gupta et al., 2019), is another possible variable that may affect study outcomes and should be taken into consideration.

Mouse models, therefore, provide valuable tools by allowing researchers to control many of these variables. Previously, we and others demonstrated that allergic sensitization to cow's milk proteins elicited behavioral abnormalities in otherwise healthy wild-type mice (de Theije et al., 2014; Germundson et al., 2018; Smith et al., 2019; Germundson et al., 2020). Moreover, these studies showed that the increases in c-Fos immunoreactivity (de Theije et al., 2014), degranulated mast cell numbers (Germundson et al., 2018), astrogliosis (Germundson et al., 2018; Smith et al., 2019), and proinflammatory cytokines (Smith et al., 2019) were found in the brains of allergen-sensitized mice. These results suggested that behavioral symptoms in food allergy were more than comorbidity, prompting further investigation of CMA as a causative factor that could influence brain function and behavior.

In our mouse model, CMA-associated behavioral changes were observed only in male mice and not in female mice (Germundson et al., 2018; Smith et al., 2019). Interestingly, sex differences have also been reported in humans for behavioral disorders

and food allergies (Altemus, 2006; Kelly and Gangur, 2009; Acker et al., 2017; Xu et al., 2018; Murray et al., 2019). Together with individual differences in offending allergen types, symptom presentations, and reaction severity, the susceptibility toward CMA-associated behavioral manifestations may depend on the genetic background of allergic individuals.

In this study, we therefore examined the influence of strain and sex differences in CMA-induced behavioral manifestations using male and female mice of two genetically distinct strains, C57BL/6J and BALB/cJ. Mice were sensitized to β-lactoglobulin (BLG: Bos d 5), a major allergen in bovine whey, for 5 weeks and challenged with BLG during the 6th week to assess their immediate physical reactions. Anxiety-like behavior and spatial memory were also tested one day after the allergen challenge. In addition, fecal microbiomes were compared among the mouse groups to determine the potential contribution of sex and strain in influencing intestinal microbiota after BLG sensitization. Our results indicated that these genetic variables significantly affected CMA sequelae and their extent, including immediate reactions to the allergen, behavior changes, systemic cytokine levels, and intestinal microbiota.

BLG Sensitization Produced Distinct Physical Responses in C57BL/6J and BALB/cJ Mouse Strains upon BLG Challenge

During the 6 weeks of sensitization, body weights of mice were recorded weekly to monitor overall health. As observed in our previous study (Smith et al., 2019), BLG sensitization had no impact on the overall growth of sex-matched mice (Figure 22A). When challenged with 50 mg of BLG in Week 6, most of the C57BL/6J mice, regardless of sex, were asymptomatic (Figure 22B) and scored 0 on the anaphylaxis scale (Table 1).

A few mice in the BLG-sensitized group showed minor symptoms although the maximum score did not exceed 2, and the differences between the average scores of sex-matched sham and BLG-sensitized groups were not statistically significant (male C57BL/6J sham: 0.10 ± 0.09, male C57BL/6J BLG: 0.6 ± 0.3, $p = 0.10$; female C57BL/6J sham: 0.10 ± 0.09, female C57BL/6J BLG 0.5 ± 0.2, $p = 0.10$; $n = 10$ in all groups). While the sensitized BALB/cJ mice of both sexes also did not score more than 2, many more animals presented with observable symptoms than sex-matched C57BL/6J mice, and the differences between the average scores of the sex-matched sham and BLG groups for BALB/cJ were significantly different (male sham: 0.10 ± 0.09, male BLG: 1.2 ± 0.3, $p = 0.0007$; female sham: 0.10 ± 0.09, female BLG: 1.8 ± 0.2, $p < 0.0001$; $n = 10$ in all groups). Sex- and treatment-matched strain differences in clinical scores were only significant between BLG sensitized female sham and BLG mice ($p < 0.0001$). Similarly, when body temperatures were measured to assess allergen-induced hypothermia (Figure 22C), the majority of BLG-sensitized male and female C57BL/6J mice maintained their normal body temperature at 30 min post-challenge, showing no significant differences between sex-matched sham and BLG groups (male C57BL/6J sham: 37.9 ± 0.2; male C57BL/6J BLG: 37.5 ± 0.4; female C57BL/6J sham: 37.75 ± 0.09; female C57BL/6J BLG: 37.3 ± 0.4; $n = 10$ in all groups). However, BLG-sensitized BALB/cJ groups of both sexes clearly presented allergen-induced hypothermia reflective of histaminergic action from mast cells (Makabe-Kobayashi et al., 2002) (male BALB/cJ sham: 38.6 ± 0.3, male BALB/cJ BLG: 36.8 ± 0.7, $p = 0.006$; female BALB/cJ sham: 37.52 ± 0.06; female BALB/cJ BLG: 35.7 ± 0.5, $p = 0.0007$; $n = 10$ in all groups). In addition, as with the clinical scores, BLG sensitized female BALB/cJ group displayed

greater hypothermic responses than sex-matched C57BL/6J mice ($p = 0.003$). In contrast, the strain differences in allergen-induced hypothermia in BLG sensitized mice were not significant in males ($p = 0.27$). These results indicated that the severity of allergic responses was sex- and strain-dependent, with female BALB/cJ mice exhibiting most robust reactions among the BLG-sensitized groups tested, followed by male BALB/cJ mice. In contrast, BLG-sensitized C57BL/6J mice displayed minimally observable physical responses upon allergen challenge as we had previously reported (Smith et al., 2019).

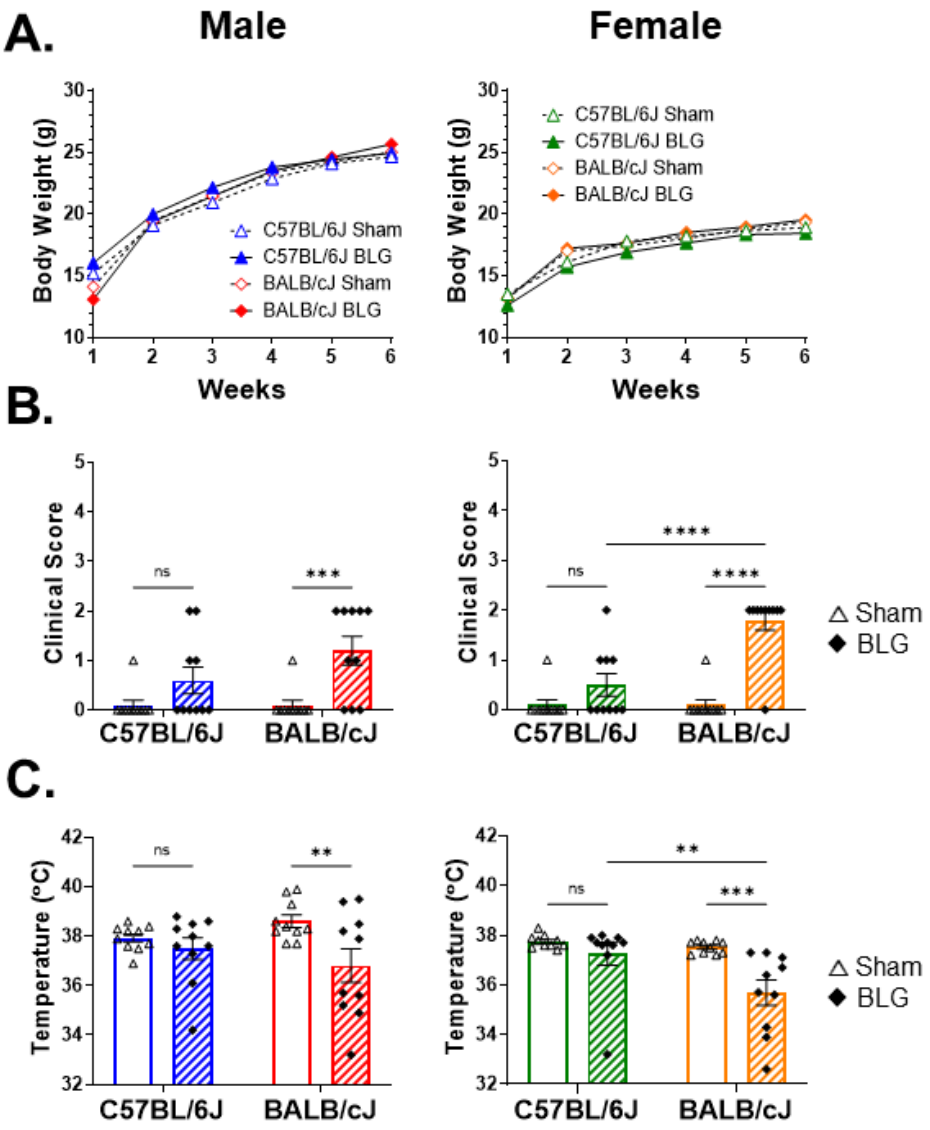


Figure 22. Physical responses of mice to BLG sensitization and challenge. (**A**) Body weight charts depicting comparable growth of male and female sham (open symbols) and BLG-sensitized (filled symbols) C57BL/6J (triangles) and BALB/cJ (diamonds) mice measured weekly during the sensitization procedure. (**B**) Clinical symptoms observed by individual mice were determined at 30 min after BLG allergen challenge in Week 6. Symptoms were scored based on the symptom score table (Table 1). (**C**) Body temperature (°C) measured at 30 min after the allergen challenge. Male C57BL/6J (blue), male BALB/cJ (red); female C57BL/6J (green), female BALB/cJ (orange). Bars in **B** and **C** indicate group average values ± SEM (two-way ANOVA), $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$, $n = 10$.

Allergen-specific Immunoglobulins were Differentially Produced in BLG-sensitized Mice in a Sex- and Strain-dependent Manner

To validate the development of adaptive immunity after the sensitization procedure, subclasses of BLG-specific immunoglobulins, IgE, IgG1, and IgG2a, were individually detected using ELISA (Figure 23, also see Supplemental Figure 3). When compared to respective sham mice, BLG-specific IgE (Figure 23A) was significantly elevated approximately by 2-fold in the sera of sensitized male C57BL/6J mice (1.8 ± 0.4 fold, $n = 10$, $p = 0.05$) and male and female BALB/cJ mice (male: 2.1 ± 0.4 fold, $n = 10$, $p = 0.006$; female: 2.20 ± 0.41 fold, $n = 10$, $p = 0.0007$), but not in BLG-sensitized female C57BL/6J mice (1.0 ± 0.2 fold, $n = 10$, $p = 0.28$). In contrast, neither male nor female C57BL/6J mice showed allergen-specific IgG1 production (Figure 23B) even though robust increases in serum levels of BLG-specific IgG1 was detected in the sensitized BALB/cJ mice of both sexes (male: 46 ± 3 fold, $n = 10$, $p < 0.0001$; female BALB/cJ BLG: 43 ± 3 fold, $n = 10$, $p < 0.0001$). Similarly, elevated levels of BLG-specific IgG2a were found in both male and female BALB/cJ mice (male: 8 ± 5 fold, $n = 10$, $p = 0.04$; female: 10 ± 3 fold, $n = 10$, $p < 0.0001$) but not in C57BL/6J mice (Figure 23C). These results indicated that BALB/cJ mice responded to BLG sensitization by productions of all immunoglobulin subclasses tested. In contrast, such response was limited to allergen-specific IgE in male C57BL/6J mice whereas females of this strain did not show significant immune responses after the 5-week sensitization regimen and an allergen challenge. Thus, the sex and strain of mice influenced the extent and the class of allergen-specific immunoglobulins produced after BLG sensitization.

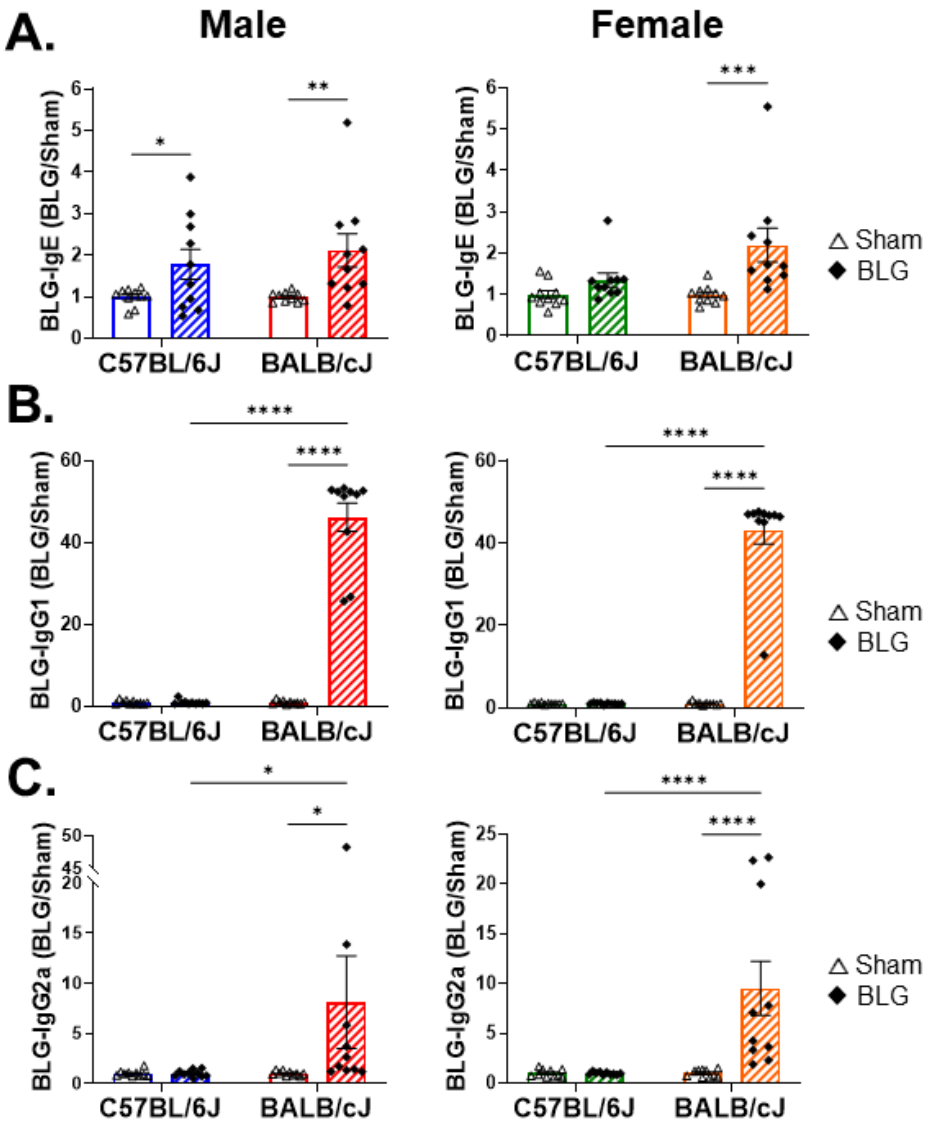


Figure 23. Serum levels of BLG-specific immunoglobulin isotypes. Terminal blood samples were used to detect BLG-specific serum IgE (**A**), IgG1 (**B**) and IgG2a (**C**) using ELISA. Fold change was calculated by normalizing optical density (OD) values obtained for BLG-sensitized groups to those for sex- and strain-matched sham groups. Sham mice (open bars with open triangles); BLG mice (striped bars with filled diamonds); male C57BL/6J (blue); male BALB/cJ (red); female C57BL/6J (green); female BALB/cJ (orange). Bars indicate group average values in fold changes ± SEM (two-way ANOVA), *$p < 0.05$, ****$p < 0.0001$, $n = 10$.

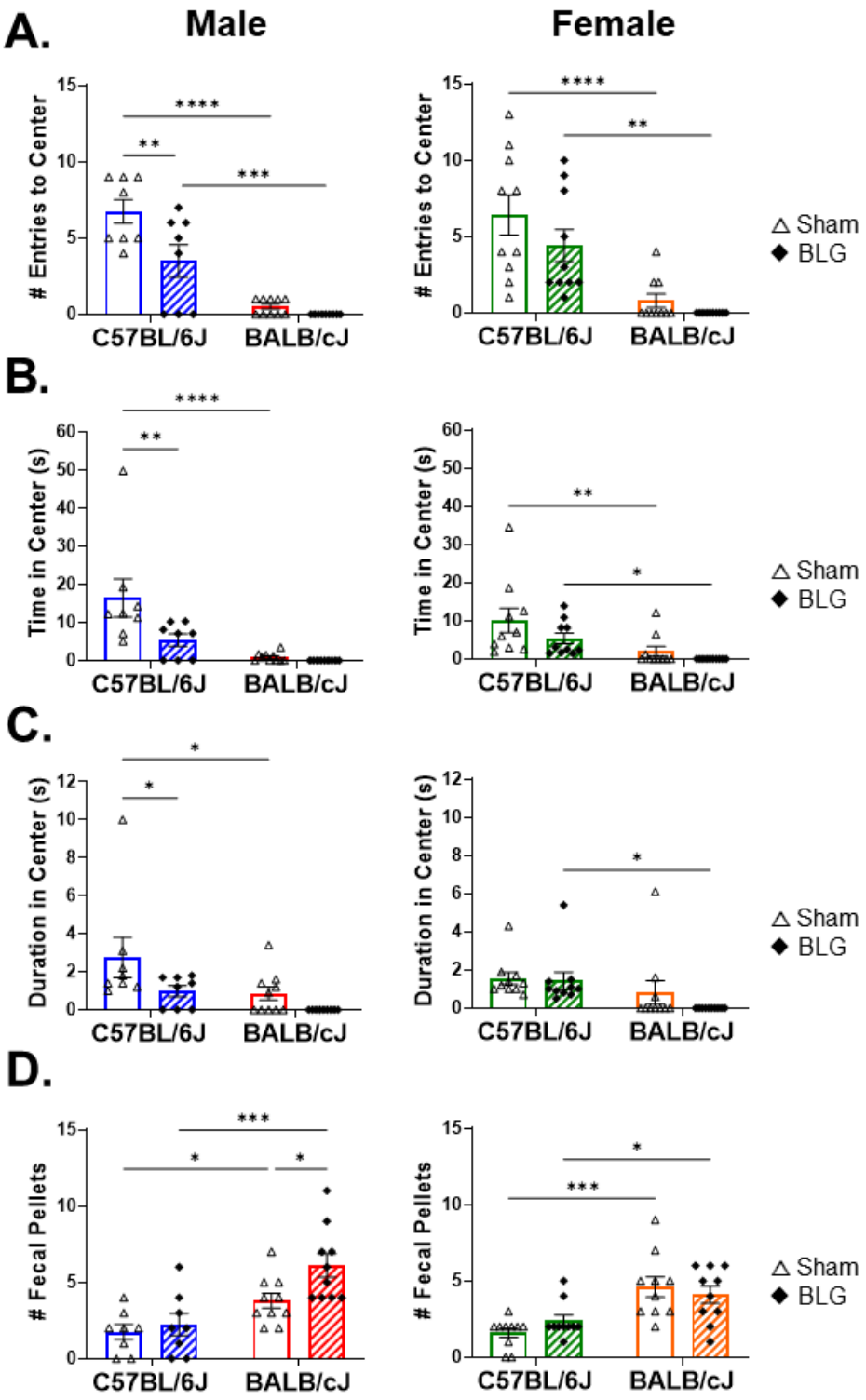
Anxiety-like Behavior Differentially Manifested in BLG-sensitized Male C57BL/6J and BALB/cJ Mice after Allergen Challenge without Affecting General Activity and Cognitive Function

For the OF test, we first tracked the frequency of visits to the center zone, the total time spent in the center zone, and the average duration of each visit to the center zone at 1-min intervals to detect possible time-dependent changes in anxiety-like behavior during the course of the 10-min testing period (Supplemental Figure 4). From this preliminary analysis, we observed that significant differences between the sham and BLG groups in the measured parameters were often apparent during the first 4 min as previously reported by others (Bailey et al., 2007; Tanda et al., 2009; Maeta et al., 2018) but became less evident during the latter half of the test duration. Thus, the activity parameters during the first 4 min of the test period were compared among the groups (Figure 24). BLG-sensitized male C57BL/6J mice made significantly fewer entries to the center zone of the OF arena (sham: 6.8 ± 0.8, n = 8; BLG: 4 ± 1, n = 8, p = 0.001), spent less total time in the center zone (sham: 16 ± 5 sec, n = 8; BLG: 5 ± 2 sec, n = 8, p = 0.004), and spent less time in the center zone per entry (sham: 3 ± 1 sec, n = 8; male C57BL/6J BLG: 1.0 ± 0.3 sec, n = 8, p = 0.03) than their sham counterparts (Figure 24A-C). The CMA-associated effects on these parameters were not statistically significant for male BALB/cJ mice and female mice of both strains. However, in comparison to C57BL/6J mice, all groups of BALB/cJ mice noticeably avoided the center zone during the test period, posing a challenge in detecting any changes with BLG sensitization. In an attempt to detect differences in anxiety-like behavior between the treatment groups for BALB/cJ mice using another approach, the numbers of fecal pellets produced during the test were counted (Crumeyrolle-Arias et al., 2014; Seibenhener and Wooten, 2015). Greater

numbers of fecal pellets, considered to reflect an increased anxiety-like state in rodents, were produced by BLG-sensitized male BALB/cJ mice than their sham control mice (Figure 24D, sham: 3.8 ± 0.5, $n = 10$; BLG: 6.1 ± 0.8, $n = 10$, $p = 0.01$) although no differences were found in any other groups.

To differentiate anxiety-like behavior from motor deficits, we also examined general locomotor activities of mice, the total distance traveled, and total time immobile. There were significant inter-strain differences between sex- and treatment-matched groups, with BALB/cJ mice, regardless of sex or treatment, being overall less active than C57BL/6J and avoiding the center zone (Supplemental Figure 5). Furthermore, BLG-sensitized male BALB/cJ mice traveled less distance than strain-matched sham mice, although the difference in time immobile was not statistically significant. In contrast, there were no significant differences between sex-matched C57BL/6J sham and BLG groups, suggesting that decreases in the number of entries to the center and time spent in the center observed in male mice of this strain were not likely due to reduced mobility.

Figure 24. Open-field activity monitoring test. Overall activity of mice in an open field arena was monitored one day after BLG challenge. The first 4 min of the test were analyzed to assess differences in the number of entries to the center area (**A**), total time spent in the center (**B)**, and the duration of each entry to the center (**C**). In addition, the number of fecal pellets excreted during the test were counted as an alternative measure of anxiety-like behavior (**D**). Sham mice (open bars with open triangles); BLG mice (striped bars with filled diamonds); male C57BL/6J (blue); male BALB/cJ (red); female C57BL/6J (green); female BALB/cJ (orange). Bars indicate group average values ± SEM (two-way ANOVA), *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, n = 8-10.


A.
Male
Female
Entries to Center
C57BL/6J
BALB/cJ
△ Sham
◆ BLG
B.
Time in Center (s)
C.
Duration in Center (s)
D.
Fecal Pellets

Anxiety-like behavior of mice was also assessed using the EZM test by monitoring their avoidance of the open zones (Figure 25). As observed with the OF test, the BALB/cJ strain exhibited overall greater tendency to avoid open zones of the EZM compared to sex- and treatment matched C57BL/6J mice. When comparing sex- and strain-matched groups, BLG-sensitized male mice of both strains made fewer entries to the open zones than their respective sham counterparts (Fig 25A; male C57BL/6J sham: 16 ± 2, n = 8; male C57BL/6J BLG: 9 ± 2, n = 8, p = 0.01; male BALB/cJ sham: 6 ± 2, n = 10; male BALB/cJ BLG: 0.6 ± 0.3, n = 10, p = 0.03). Similarly, BLG-sensitized male C57BL/6J and BALB/cJ mice spent less time in the open zone than sex- and strain-matched sham mice (Fig 25B; male C57BL/6J sham: 82 ± 9, n = 8; male C57BL/6J BLG: 42 ± 12, n = 8, p = 0.006; male BALB/cJ sham: 26 ± 11, n = 10; male BALB/cJ BLG: 0.9 ± 0.4, n = 10, p = 0.05). In addition, sensitized BALB/cJ male mice also spent less time in the open zone per entry than sham mice (Fig 25C; male BALB/cJ sham: 2.5 ± 0.8, n = 10; male BALB/cJ BLG: 0.7 ± 0.4, n = 10, p = 0.03). No differences were found in female mice of either strain in these parameters. These results further supported that sex and strain influenced the manifestation of CMA-associated anxiety-like behavior.

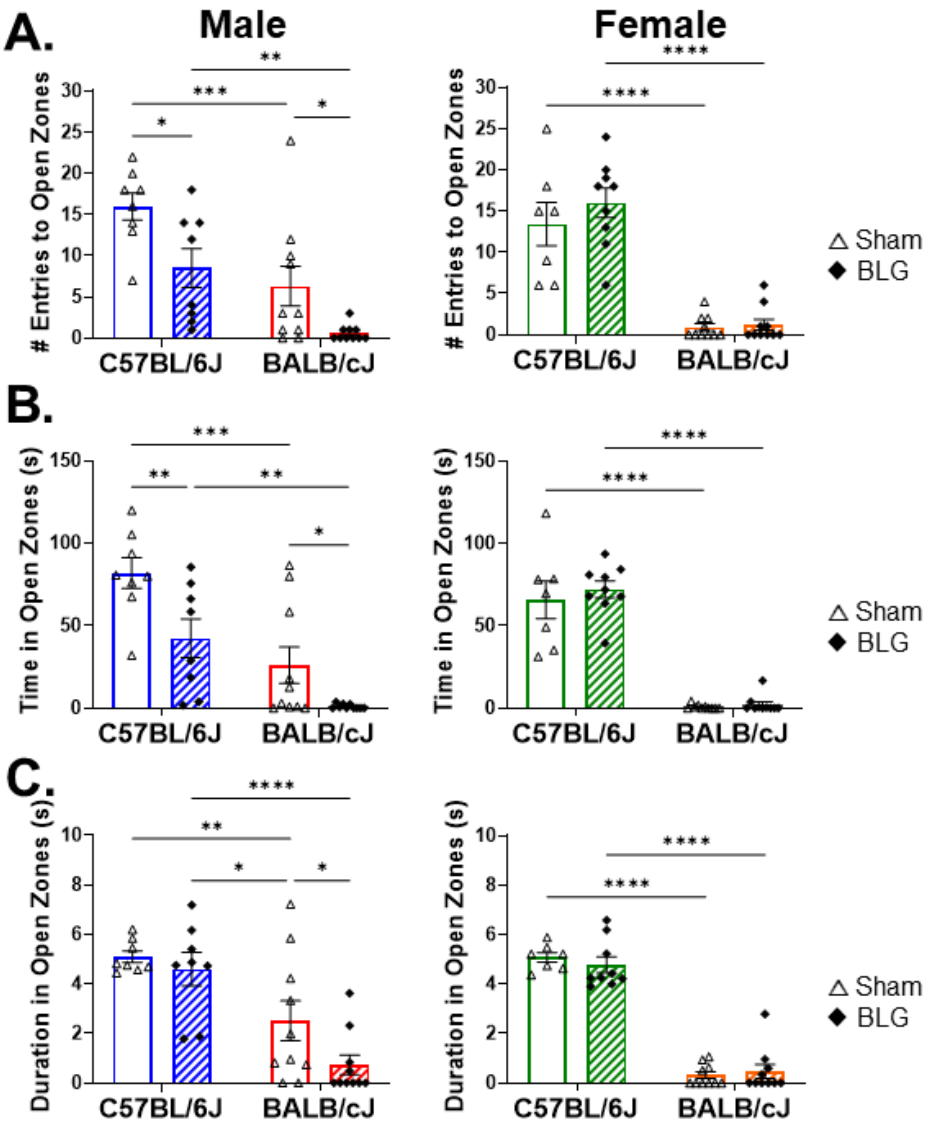


Figure 25. Elevated zero maze test. Anxiety-like behavior of mice was observed on an EZM apparatus one day after BLG challenge. The number of entries to the open zones (**A**), total time spent in the open zones (**B**), and the duration of each entry to the open zones (**C**) were quantified. Sham mice (open bars with open triangles); BLG mice (striped bars with filled diamonds); male C57BL/6J (blue); male BALB/cJ (red); female C57BL/6J (green); female BALB/cJ (orange). Bars indicate group average values ± SEM (two-way ANOVA), *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, n = 7-10.

To evaluate whether CMA altered cognitive function, spatial memory was tested by quantifying the number of spontaneous alternations performed in the cross-maze test. In contrast to anxiety-like behavior, the ability of mice to strategically explore each arm of the maze was not affected by BLG sensitization in either strain or sex (Figure 26A). However, it is important to note that many of the female BALB/cJ mice (5 sham and 6 BLG mice) either stayed in the entry arm or did not complete a cycle into all arms during their test period (Fig 26B) and were therefore excluded from the final analysis of % alternations (Figure 26A). These results suggested that BLG sensitization did not affect cognitive ability with respect to spatial memory. No obvious strain differences were detected in their ability to alternately explore all arms of the maze.

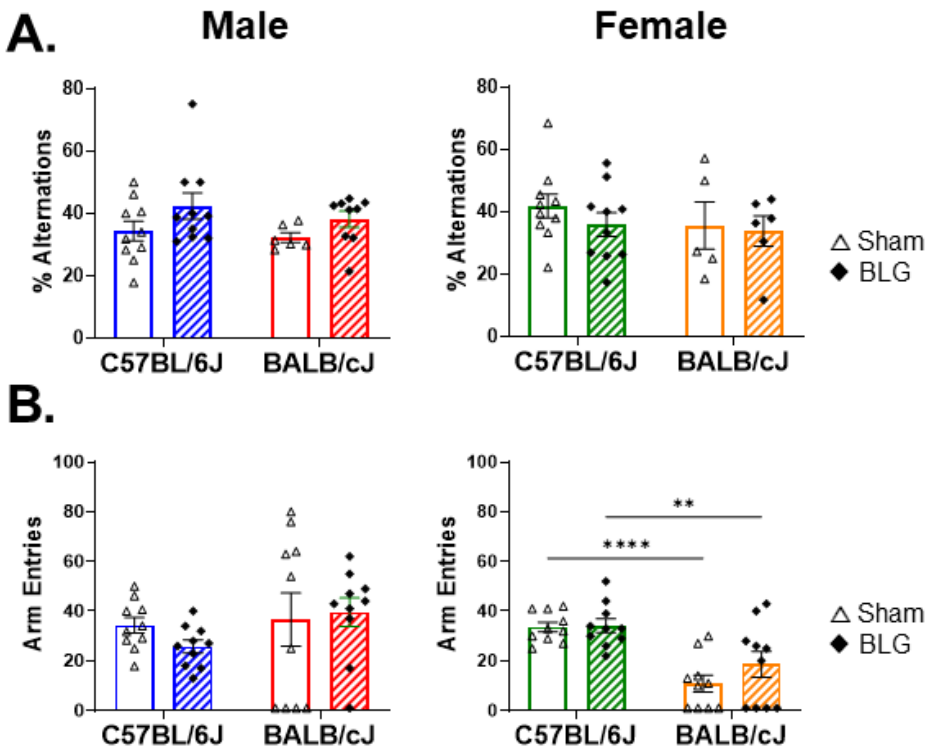


Figure 26. Cross maze test. Spatial memory of mice was tested using a cross maze one day after BLG challenge. Mice were allowed to explore the maze freely for 12 min, and the numbers of successful sequence alternations (**A**) and total number of entries into the arms (**B**) were recorded manually from video files. The number of successful alternations of arm entries made by each mouse was converted to percent alternations using the equation described in the Materials and Methods section. Mice that did not leave the starting position were removed from the final analysis. Sham mice (open bars with open triangles); BLG mice (striped bars with filled diamonds); male C57BL/6J (blue); male BALB/cJ (red); female C57BL/6J (green); female BALB/cJ (orange). Bars indicate group average values ± SEM (two-way ANOVA), $^{**}p < 0.01$, $^{****}p < 0.0001$, $n = 5\text{-}10$.

BLG Sensitization Yielded Distinct Sex- and Strain-dependent Plasma Cytokine and Chemokine Profiles

Because CMA-induced immunoglobulin production and anxiety-like behavior manifestation were sex- and strain-dependent, it was likely that distinct immune responses with unique inflammatory mediators were triggered in each mouse group upon allergen challenge. Thus, we next characterized cytokines, chemokines, and associated immunologic factors in each experimental group. In particular, we expected to observe elevation of Th1- and Th2-associated cytokines in C57BL/6J and BALB/cJ strains as they reportedly have respective immune biases (Autenrieth et al., 1994; Nishimura et al., 1997; Mills et al., 2000; Watanabe et al., 2004). To quantify the factors at the systemic level, we assessed the plasma samples using Quantibody® Mouse Cytokine Array Q5 system (Figure 27, see Supplemental Figure 6 for the complete quantitative data). In BLG-sensitized male C57BL/6J mice, 9 analytes were significantly increased in comparison to their respective sham mice (Figure 27A). CCL1 (C-C motif chemokine ligand 1) and CSF1 (colony stimulating factor 1, also known as macrophage colony stimulating factor or M-CSF) showed the most striking changes with allergen sensitization by increasing 12 ± 4 fold and 9 ± 2 fold, respectively. However, the absolute amounts of these factors were relatively low (CCL1 in sham: 1.1 ± 0.5 pg/mL, BLG: 13 ± 4 pg/mL; $n = 10$, $p = 0.0003$; CSF1 in sham: 19 ± 10 pg/mL, BLG: 176 ± 38 pg/mL; $n = 10$, $p = 0.0001$). Other analytes that were significantly induced in sensitized male C57BL/6J mice were, in the order of greatest to lowest, 7 ± 2 fold for IL-13 (sham: 780 ± 261 pg/mL, BLG: 5353 ± 1667 pg/mL; $n = 10$, $p = 0.0007$), 6 422 ± 2 fold for CCL17 (sham: 83 ± 22 pg/mL, BLG: 528 ± 187 pg/mL; $n = 10$, $p = 0.004$), 4.5 ± 0.8 423 fold for IL-21 (sham: 41 ± 23 pg/mL, BLG: 188 ± 33 pg/mL; $n = 10$, $p = 0.0009$),

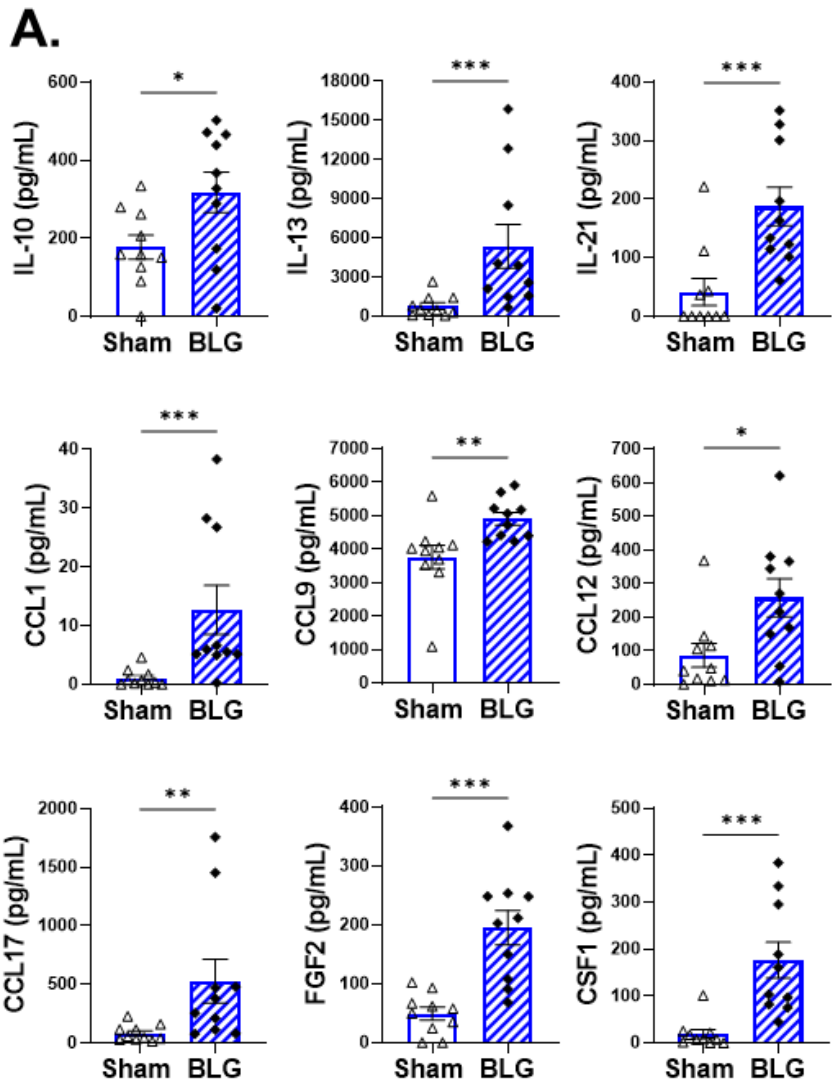
4.0 ± 0.6 fold for FGF2 (Fibroblast growth factor 2;sham: 49 ± 11 pg/mL, BLG: 195 ± 29 pg/mL; $n = 10$, $p = 0.0001$), 3.0 ± 0.7 fold for CCL12 (sham: 86 ± 35 pg/mL, BLG: 258 ± 57 pg/mL; $n = 10$, $p = 0.02$), 1.8 ± 0.3 fold for IL-10 (sham: 177 ± 31 pg/mL, BLG: 318 ± 52 pg/mL; $n = 10$, $p = 0.04$), and 1.31 ± 0.05 fold for CCL9 (sham: 3762.313 ± 355 pg/mL, BLG: 4910 ± 191 pg/mL; $n = 10$, $p = 0.002$). None of the analytes quantified with this assay were induced in BLG-sensitized female C57BL/6J mice (see Supplemental Figure 6).

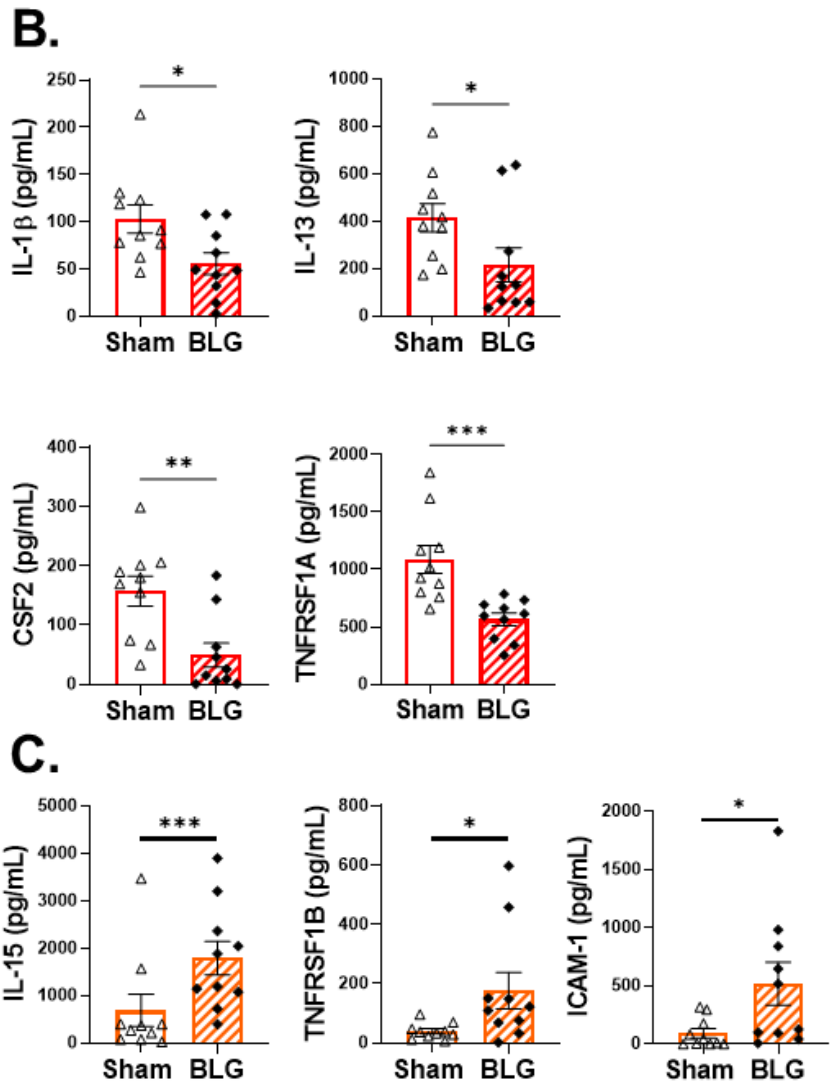
The cytokine profile of BLG-sensitized male BALB/cJ mice was distinct from that of sex matched C57BL/6J mice, and 4 analytes were significantly reduced in BLG-sensitized mice compared to the sham mice (Figure 27B). The analyte levels were lower by 0.5 ± 0.1 fold for IL-1β (sham: 103 ± 15 pg/mL, BLG: 56 ± 11 pg/mL, $n = 10$, $p = 0.02$), 0.5 ± 0.2 fold for IL-13 (sham: 414 ± 59 pg/mL, BLG: 216 ± 72 pg/mL; $n = 10$, $p = 0.03$) 0.3 ± 0.1 fold for CSF2 (sham: 157 ± 25 pg/mL, BLG: 49 ± 20 pg/mL, $n = 10$, $p = 0.002$), and 0.52 ± 0.05 fold for TNFRSF1A (sham: 1087 ± 121 pg/mL, BLG: 566 ± 56 pg/mL; $n = 10$, $p = 0.0002$) in BLG sensitized mice. In contrast, 3 analytes were increased in BLG-sensitized female BALB/cJ mice (Figure 27C), including 2.6 ± 0.5 fold for IL-15 (sham: 683 ± 340 pg/mL, BLG: 1788 ± 353 pg/mL, $n = 10$, $p = 0.007$), 5 ± 2 fold for TNFRSF1B (sham: 39 ± 8 pg/mL, BLG: 176 ± 61 pg/mL, $n = 10$, $p = 0.02$), and 6 ± 2 fold for ICAM-1 (sham: 93 ± 40 pg/mL, BLG: 518 ± 184 pg/mL, $n = 10$, $p = 0.02$).

This array data demonstrated that BLG sensitization resulted in altered levels of distinct sets of immunologic mediators in the circulation, with C57BL/6J male mice having the greatest number of mediators that were significantly affected. Therefore, our results indicated that the same allergen triggered varying immune responses depending

on the sex and strain of sensitized mice. Importantly, the cytokine profiles from BLG-sensitized C57BL/6J and BALB/cJ mice failed to categorize their strain-specific responses simply into Th1 and Th2 responses, respectively.

Figure 27. Plasma levels of immune mediators that were significantly different between sex- and strain matched sham and BLG-sensitized mice. Levels of the 40 immune mediators included in the Quantibody Mouse Cytokine Array 5 (QAM-CYT-5) were quantified from plasma samples. Only the analytes that showed significant differences between sex- and strain-matched sham and BLG groups are shown for C57BL/6J male mice (**A**), BALB/cJ male mice (**B**), and BALB/cJ female mice (**C**). No significant differences in any of the detected analytes were observed in female C57BL/6J mice (not shown). The quantification of all analytes for all mouse groups are presented as Supplemental Figure 6. Sham mice (open bars with open triangles); BLG mice (striped bars with filled diamonds); male C57BL/6J (**A**); male BALB/cJ (**B**); female BALB/cJ (**C**). Bars indicate group average values ± SEM (Mann-Whitney), $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $n = 10$.


A.
IL-10 (pg/mL)
IL-13 (pg/mL)
IL-21 (pg/mL)
CCL1 (pg/mL)
CCL9 (pg/mL)
CCL12 (pg/mL)
CCL17 (pg/mL)
FGF2 (pg/mL)
CSF1 (pg/mL)
Sham
BLG


B.
IL-1β (pg/mL)
250
200
150
100
50
0
*
Sham
BLG
IL-13 (pg/mL)
1000
800
600
400
200
0
*
Sham
BLG
CSF2 (pg/mL)
400
300
200
100
0
**
Sham
BLG
TNFRSF1A (pg/mL)
2000
1500
1000
500
0
***
Sham
BLG
C.
IL-15 (pg/mL)
5000
4000
3000
2000
1000
0
***
Sham
BLG
TNFRSF1B (pg/mL)
800
600
400
200
0
*
Sham
BLG
ICAM-1 (pg/mL)
2000
1500
1000
500
0
*
Sham
BLG

BLG-sensitization Differentially Altered the Composition of Intestinal Microbial Community in a Sex- and Strain-specific Manner

Because BLG was orally introduced during the sensitization procedure, we postulated that allergen sensitization and the subsequent challenge had produced inflammatory conditions in the intestinal tract and influenced the commensal microbial community. Altered intestinal microbiota has been implicated in a variety of pathological conditions, including food allergy and neuropsychiatric disorders (Wang et al., 2011; Scheperjans et al., 2015; Blazquez and Berin, 2017; Vuong et al., 2017; Pulikkan et al., 2018). To assess whether BLG sensitization resulted in alterations in intestinal microbiota, we next performed microbiome analysis by 16S ribosomal RNA gene sequencing from stool samples. Following sequencing, microbial taxonomy was classified using amplicon sequence variants (ASVs) at the species level. Approximately 400-500 species were classified in each treatment group (Figure 28A). There was a significant strain dependent difference in the number of observed species between C57BL/6J and BALB/cJ male sham mice (Figure 28A). Species richness was greater for BALB/cJ sham mice compared to respective C57BL/6J mice in males (C57BL/6J sham: 392.2 ± 21.3, BALB/cJ sham: 500.3 ± 17.0; $n = 10$, $p < 0.0001$). Furthermore, assessment of alpha diversity indicated significant differences between sex- and strain-matched treatment groups for male C57BL/6J mice (Simpson index) and female BALB/cJ mice (Shannon and Simpson indices), showing BLG associated decrease and increase in biodiversity, respectively (Supplemental Figure 7).

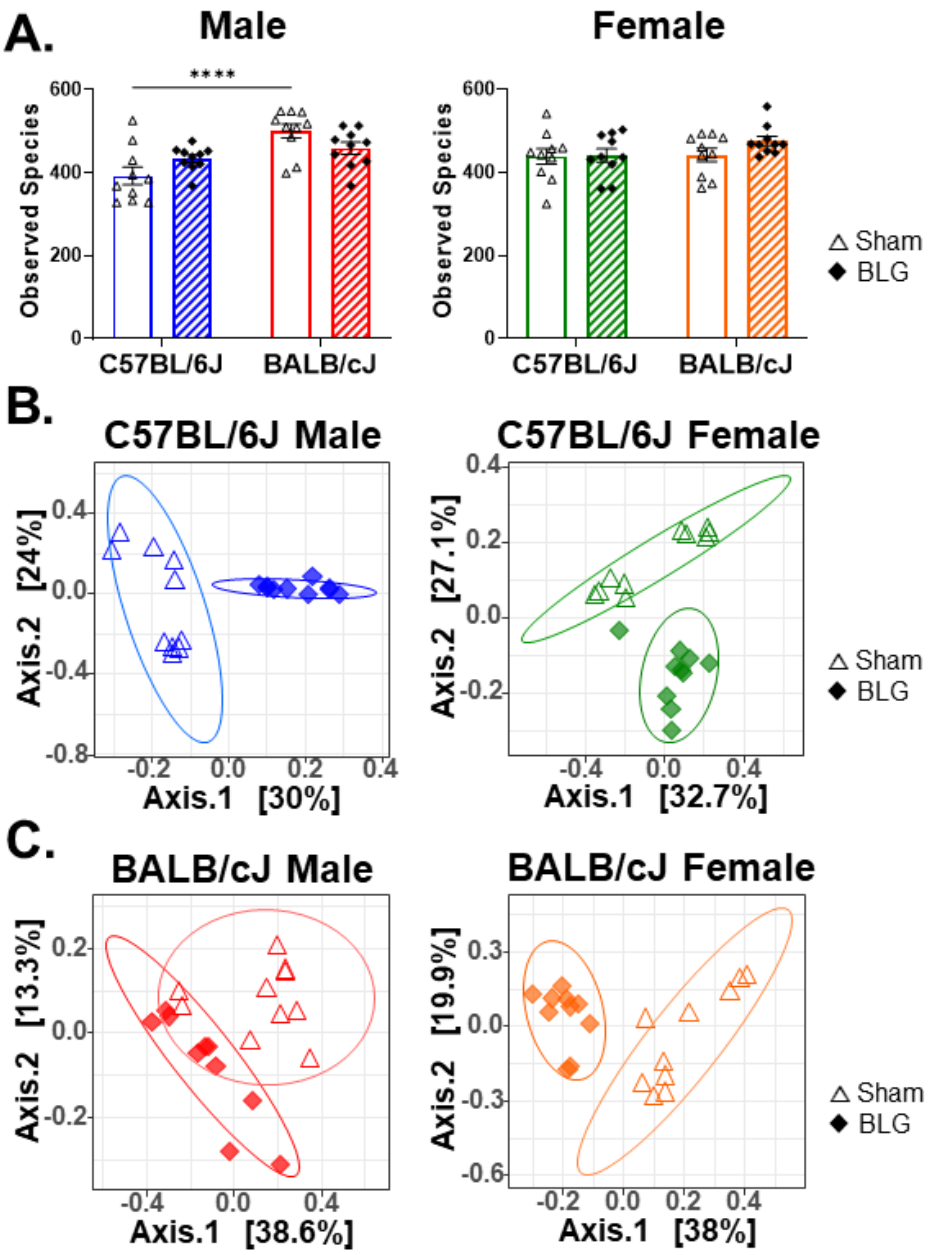
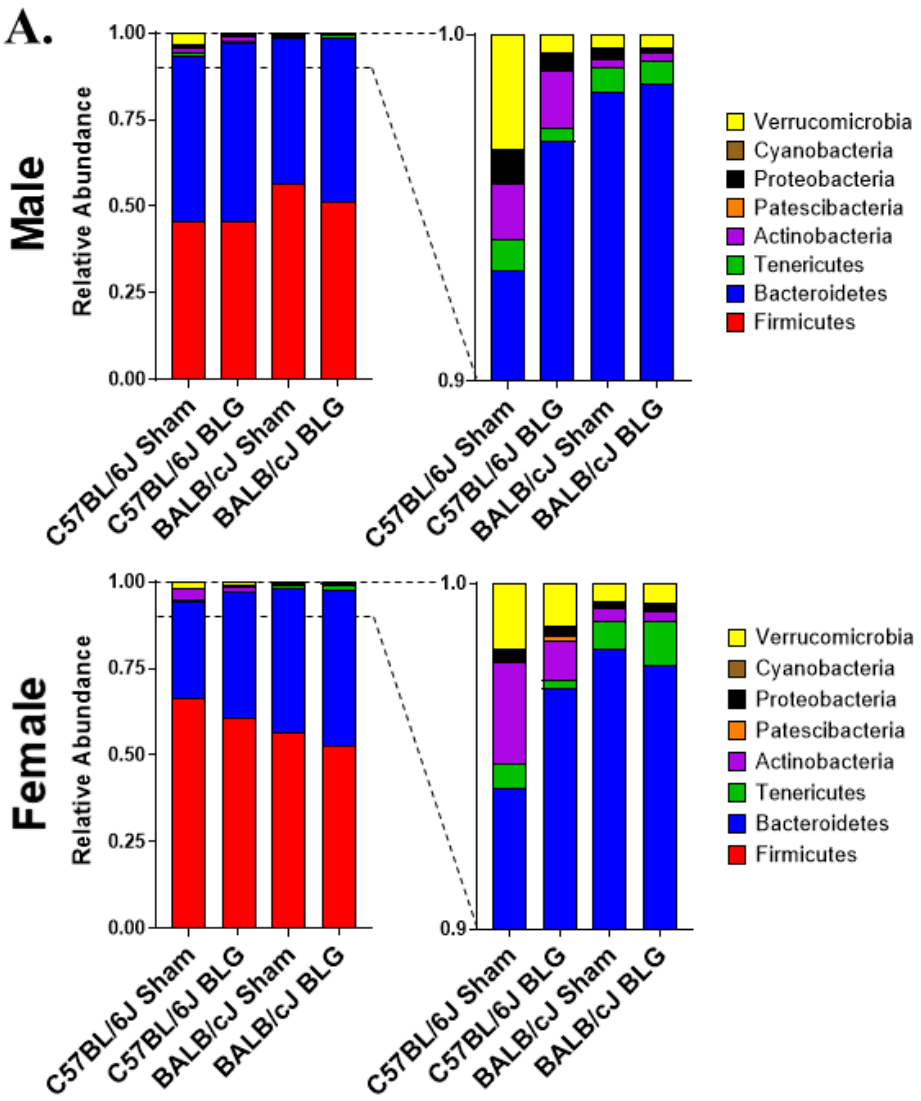


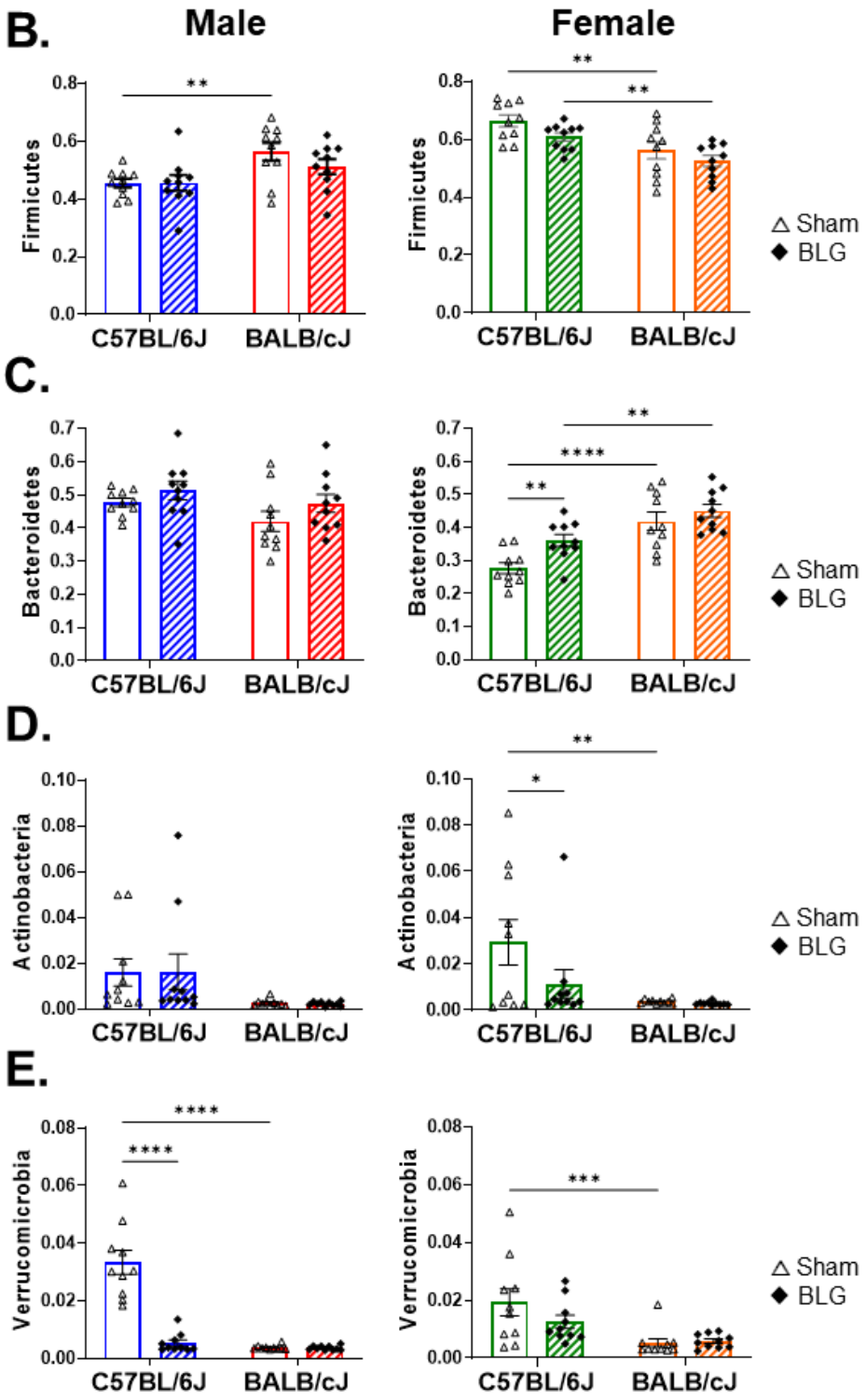
Figure 28. Effects of BLG sensitization on fecal microbiome. Microbial DNA was isolated from fecal pellets and 16S ribosomal RNA gene sequencing was performed. Fecal microbial compositions were determined as described in the Materials and Methods section, and alpha and beta diversities were assessed. (**A**) Assessment of alpha diversity with the number of observed species. Sham mice (open bars with open triangles); BLG mice (striped bars with filled diamonds). Bars indicate group average values ± SEM (two-way ANOVA), ****$p < 0.0001$, $n = 10$. (**B, C**) Assessment of beta diversity with Bray-Curtis principal coordinate analysis. Sham mice (open triangles); BLG mice (filled diamonds). Male C57BL/6J (blue); male BALB/cJ (red); female C57BL/6J (green); female BALB/cJ (orange).

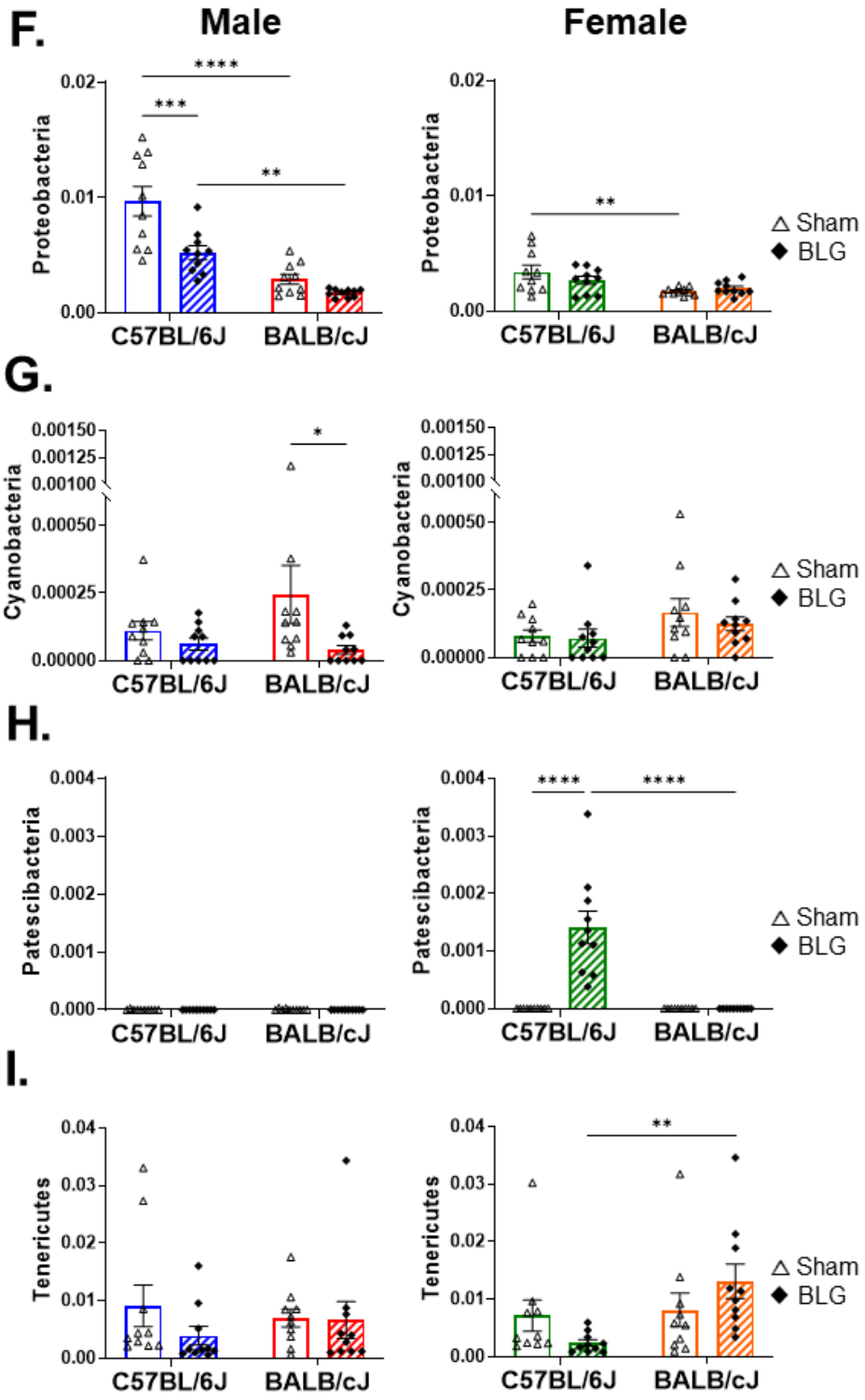
We next performed PCoA using the Bray-Curtis dissimilarity method to cluster the beta diversity of sex- and strain-matched treatment groups. There was clear separation between sham and BLG groups in both male and female C57BL/6J mice (Figure 28B). Similarly, the clustering of the two groups was distinct for female BALB/cJ mice, whereas some overlap was observed for male BALB/cJ mice (Figure 28C). These results suggested that intestinal microbial composition becomes altered with BLG sensitization, although the extent of the change may be strain-dependent.

When taxonomic compositions from the experimental groups were compared at the phylum level, each group showed a distinct profile, although Firmicutes and Bacteroidetes were the two dominant bacterial phyla as reported elsewhere (Figure 29A). The relative abundance of these phyla was uniquely influenced by BLG sensitization in a sex- and strain-dependent manner (Figure 29B-I). For C57BL/6J, Verrucomicrobia and Proteobacteria were significantly lower in BLG-sensitized male mice, whereas sensitization resulted in a greater abundance of Bacteroidetes and Patescibacteria and reduced abundance of Actinobacteria in female mice. In contrast, for BALB/cJ mice, Cyanobacteria was the only phylum that was lower in BLG-sensitized male mice, while female mice did not show any significant differences between sham and BLG groups in the identified phyla. Among the differences described above, the most notable sensitization associated differences were reduced abundance of Verrucomicrobia in male C57BL/6J by 84.1% (0.16 ± 0.03 fold) and increased abundance of Bacteroidetes in female C57BL/6J by 30.5% (1.31 ± 0.07 fold). The complete microbiome profiles for all the experimental groups are provided in Supplemental Figure 9.

Figure 29. Sensitization-associated differences in the relative abundance of major bacterial phyla detected from fecal microbiome analysis. Microbial DNA was isolated from fecal pellets and 16S ribosomal RNA gene sequencing was performed. (**A**) Relative abundances of detected bacterial phyla were compared among sex-matched groups. (**B-I**) Relative abundance of each of the major phyla was compared to their sex-matched groups. Sham mice (open bars with open triangles); BLG mice (striped bars with filled diamonds); male C57BL/6J (blue); male BALB/cJ (red); female C57BL/6J (green) female BALB/cJ (orange). Bars indicate group average values ± SEM (two-way ANOVA), *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, $n = 10$.


A.
Male
Relative Abundance
1.00
0.75
0.50
0.25
0.00
1.0
0.9
C57BL/6J Sham
C57BL/6J BLG
BALB/cJ Sham
BALB/cJ BLG
Verrucomicrobia
Cyanobacteria
Proteobacteria
Patescibacteria
Actinobacteria
Tenericutes
Bacteroidetes
Firmicutes
Female
Relative Abundance
1.00
0.75
0.50
0.25
0.00
1.0
0.9
C57BL/6J Sham
C57BL/6J BLG
BALB/cJ Sham
BALB/cJ BLG
Verrucomicrobia
Cyanobacteria
Proteobacteria
Patescibacteria
Actinobacteria
Tenericutes
Bacteroidetes
Firmicutes


B.
Male
Female
Firmicutes
C.
Bacteroidetes
D.
Actinobacteria
E.
Verrucomicrobia
C57BL/6J
BALB/cJ
△ Sham
◆ BLG


F.
Male
Female
Proteobacteria
0.02
0.01
0.00
****
***
**
**
C57BL/6J
BALB/cJ
△ Sham
◆ BLG
G.
Cyanobacteria
0.00150
0.00125
0.00100
0.00050
0.00025
0.00000
*
H.
Patescibacteria
0.004
0.003
0.002
0.001
0.000
****
****
I.
Tenericutes
0.04
0.03
0.02
0.01
0.00
**

Proliferation of *Akkermansia muciniphila* in C57BL/6J Male Mice was Inhibited with BLG Sensitization

Because C57BL/6J male mice exhibited significant changes in intestinal microbiota associated with anxiety-like behavior, we focused on this group to investigate the potential role of their commensal bacteria in their behavioral and immunologic responses. Here, we examined the pre- and post-sensitization fecal amounts of *Akkermansia muciniphila*, a species belonging to the phylum, Verrucomicrobia, which was significantly decreased in BLG-sensitized mice (Figure 29E). *A. muciniphila* has also been implicated in various disease conditions, including neurological disorders (Wang et al., 2011; Hill-Burns et al., 2017; Li et al., 2019; Xu et al., 2019). As shown in Figure 30, the relative amounts of *A. muciniphila* detected in pre-sensitization samples from the sham and BLG groups were comparable, ranging from 4.5×10^{-7} to 1.1×10^{-5} (sham average: $4\times10^{-6} \pm 1\times10^{-6}$, BLG average: $3.2\times10^{-6} \pm 0.7\times10^{-6}$; expressed as 2^{-Cq} values). However, *A. muciniphila* increased in sham mice during the course of the 5-week sensitization period, averaging 22 ± 8 fold increases at post-sensitization with a range of 2.2 to 5-fold increases. On the other hand, all BLG-sensitized mice, except one mouse identified as an outlier (2^{-Cq} value: 9.4×10^{-6}, see the Materials and Methods section), showed profound decreases in the relative amount of the bacteria after sensitization, averaging 0.2 ± 0.1 fold, indicating that the growth of this bacteria was stifled in this group. Because *A. muciniphila* was the predominant species of Verrucomicrobia identified and detected in the microbiome (Supplemental Figure 9), the significant reduction of the phylum observed in BLG-sensitized C57BL/6J male mice is therefore likely due to attenuated colonization of *A. muciniphila* (Figure 29A).

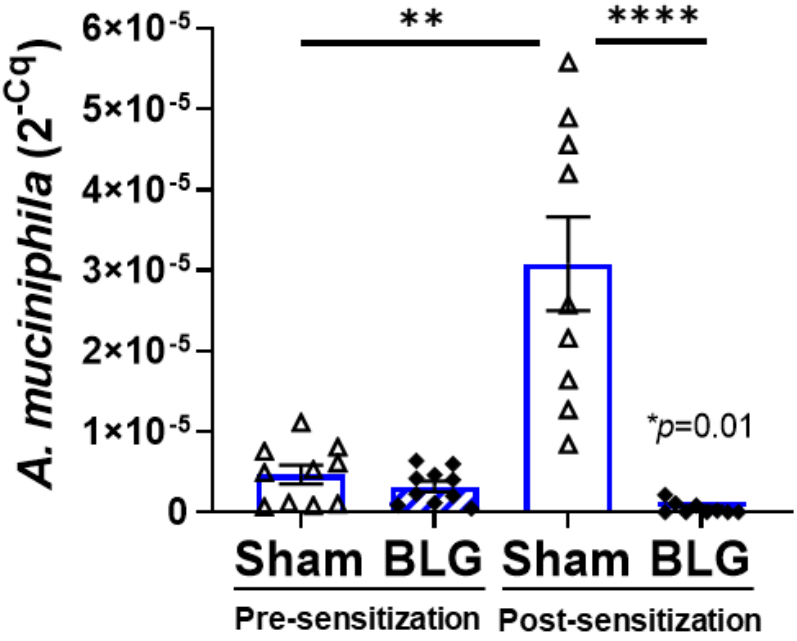


Figure 30. Differences in the amount of *A. muciniphila* in sham and BLG-sensitized male C57BL/6J mice before and after the sensitization procedure. Microbial DNA samples were isolated from fecal pellets that had been collected before (pre-sensitization) and after (post-sensitization) the 5-week sensitization procedure. The amount of *A. muciniphila* in each of the DNA samples were quantified by qPCR using a specific primer pair. Sham mice (open bars with open triangles); BLG mice (striped bars with filled diamonds). Bars indicate group averages of individual 2^{-Cq} values ± SEM, $^{**}p < 0.01$, $^{****}p < 0.0001$, $n = 10$., sham vs. BLG: two-way ANOVA; treatment-matched pre- vs post-sensitization values were compared using paired t-test. $^{*}p=0.01$ indicates comparison between pre- and post-sensitization BLG.

The Altered Microbiome Profile of BLG-sensitized Male C57BL/6J was Associated with Molecular Interactions Known to Affect Neurological Functions

In order to explore possible molecular targets that might have been influenced in our BLG sensitized mice, we identified the pathways that were associated with the microbial changes observed in this study using the Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway Database (https://www.genome.jp/kegg/pathway.html). Known molecular interaction pathways with significant differences between sex- and strain-matched sham and BLG-sensitized groups are presented as Supplemental Figure 8. We found a greater number of pathways that were significantly associated with the microbiome of BLG-sensitized C57BL/6J male mice than any other groups. Interestingly, among them were some pathways involved in neurological disorders and neurotransmission, such as serotonergic/dopaminergic synapses and Parkinson's disease (Figure 31). Although the analysis does not articulate specific molecules within each pathway that were likely affected by the sensitization-associated microbiome changes in C57BL/6J male mice, the result suggests that the neurological functions were possibly influenced in these mice.

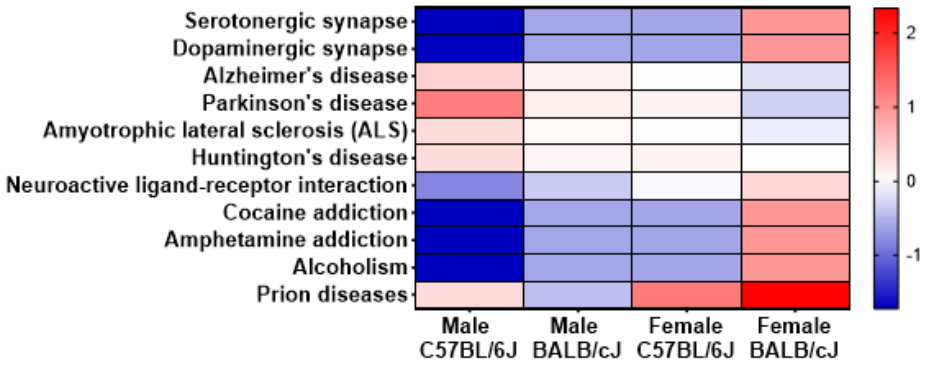


Figure 31. Central nervous system-related pathways associated with the changes in microbiota in BLG-sensitized mice. KEGG pathway analysis was used to identify known functional pathways that were significantly associated with the microbiome profiles in our mouse groups. Only the pathways that are related to the central nervous system functions are shown. All results from the KEGG pathway analysis are provided in Supplemental Figure 8.

CHAPTER VI

DISCUSSION

Study 1 – Astrogliosis Associated with Behavioral Abnormality in a Non-anaphylactic Mouse Model of Cow's Milk Allergy

For several decades, the association of FAH with behavioral, emotional and cognitive impairments has been suggested, often referred to as "cerebral allergy" (Davison, 1949) or "allergic tension-fatigue syndrome" (Speer, 1954, 1958). More recently, a growing number of reports have more specifically described FAH comorbidities with depression (Patten and Williams, 2007; Garg and Silverberg, 2014; Ferro et al., 2016), anxiety (Lyons and Forde, 2004; Patten and Williams, 2007; Garg and Silverberg, 2014; Shanahan et al., 2014; Ferro et al., 2016), ADHD (Garg and Silverberg, 2014; Shanahan et al., 2014; Ferro et al., 2016; Topal et al., 2016), and autism (Lyall et al., 2015; Xu et al., 2018). However, the evidence that FAH in fact modifies physiological functions of the brain is still insufficient, and the mechanism remains to be elucidated.

One of the major obstacles in the assessment of brain pathophysiology in FAH-associated neuropsychiatric conditions is controlling the variables associated with the study subjects, such as genetic background, diet, socioeconomic status, local environment, and culture, all of which may contribute to differences in behavior as well as FAH development. In addition, experimental parameters for quantitative assessments are often limited to evaluation scores on questionnaires for neuropsychiatric conditions

and blood IgE levels and/or skin tests for FAH. While it is undeniably challenging to evaluate mood- and emotion-elicited behavior in animal models, performing a series of behavioral tests helps to validate the results. Furthermore, animal models provide many advantages in an experimental study by allowing to control genetic, environmental, and dietary variables and to directly evaluate pathophysiology of the brain and other organs. Indeed, a mouse model of CMA with the C3H/HeOuJ strain has been utilized to demonstrate autistic-like deficit in social behavior and neurochemical changes in the brain (de Theije et al., 2014).

In the present study, we produced non-anaphylactic CMA in C57BL/6J mice using BLG as the allergen and assessed the cellular and molecular changes in the intestine and brain to identify CMA-induced pathology that might have contributed to their abnormal behavioral outcomes. During the 7 weeks of the sensitization/challenge period, both male and female mice showed no differences in their growth rate (Figure 4A). Importantly, we did not observe overt anaphylaxis symptoms in any of the groups after each of the two challenges at Week 6 and 7, although significant increases in BLG-specific IgE and IgG1 levels were observed in both male and female sensitized mice (Figures 4B, C). This result indicated that acquired immunity to BLG can be established without observable physical reactions.

Our behavioral assessments of sham and BLG-sensitized mice included digging and grooming frequencies, EZM, and TST (Figure 5). Digging behavior in rodents is an innate burrowing behavior, and the test is often performed by placing the animal in a cage with a thick layer of bedding without or with marbles (Deacon, 2006). Unlike the WP-sensitized mice we previously described (Germundson et al., 2018), BLG-sensitized mice

did not exhibit decreased digging behavior. Instead, there was an increased trend in male sensitized mice, suggesting that the behavioral effect of BLG sensitization appears to be distinct from that of the WP mixture. Although the reason for the discrepancy between the two mouse models of CMA in this behavioral outcome is not clear, it may be postulated that other constituents in the WP mixture, such as α-lactalbumin, immunoglobulins, and lactoferrin (Farrell et al., 2004), had a more diverse effect than BLG alone.

Grooming is another intrinsic rodent behavior consisting of a complex series of movements, and the frequency, total time spent, and sequence of grooming can be affected by extrinsic factors such as stress (Kalueff et al., 2016). We observed that female sham mice groomed more frequently than their male counterpart (Figure 5C), and the frequency of female grooming behavior was not affected by BLG sensitization. On the contrary, BLG-sensitized male mice showed significantly elevated grooming behavior, indicative of their stressed or anxious state (Kalueff et al., 2016). This observation was corroborated by their performance in the EZM test, in which male BLG-sensitized mice spent significantly less time than the sham mice in the open zones when entered (Figure 5B). These results together support the notion that BLG-sensitized male mice exhibited anxiety-like behavior.

Because anxiety and depression are often comorbid (Johansson et al., 2013; Tiller, 2013), we also examined whether the BLG-sensitized mice would exhibit depression-like behavior. In the TST, depression-like behavior is quantified by the animal's immobility, which reflects decreased attempts to escape from the helpless position (Cryan et al., 2005). Our results demonstrated that male BLG-sensitized mice indeed displayed

depression-like behavior, although no difference was observed between sham and BLG-sensitized female mice (Figure 5D). From our observation that the overall activity of male and female mice did not differ between sham and BLG-sensitized groups, it is unlikely that the immobility resulted from inability to move or lethargy (Figure 5E). Taken together, our behavioral tests indicated that BLG sensitization elicited anxiety- and depression-like behavior in male-specific manner. This sex-dependent behavior manifestation was also observed with WP-sensitized mice and have been discussed previously (Germundson et al., 2018).

Interestingly, similar sex differences in behavioral observations have been reported with human patients with neuropsychiatric disorders, including ADHD, obsessive-compulsive disorder (OCD), and autism spectrum disorder (ASD). Several meta-analysis studies have indeed found greater prevalence in male population (Hanna, 1995; Gaub and Carlson, 1997; Gershon, 2002; Mathis et al., 2011; Russell et al., 2011). This male dominance in these conditions seem to arise from the fact that male patients exhibit more noticeable behavioral phenotypes than female patients. For example, boys with ADHD display more externalized and/or disruptive behavior than girls, who in contrast tend to show more internalized, inattentive behavior (Gaub and Carlson, 1997; Gershon, 2002). Similarly, some studies on sexual dimorphism in ASD symptomatology reported that boys have more severe autistic traits and therefore are more likely to be diagnosed with ASD than girls (Russell et al., 2011; Mandy et al., 2012). Biological factors, such as sex hormone-dependent structural development of the prefrontal and orbitofrontal cortices, thalamus, and basal ganglia (Maia et al., 2008), the volume of the pituitary gland (MacMaster et al., 2006), and polymorphisms in the serotonergic system

(Mathis et al., 2011; Verma et al., 2014; Shuffrey et al., 2017), have been suggested to underlie the sex differences in behavioral manifestations.

In addition to behavioral differences, sexual dimorphism of the immune systems has been well-recognized. Gene regulation by gonadal hormones and the expression of X-chromosome genes are known to differentially affect the immune system in males and females, including immunoglobulin productions, T-lymphocyte functions and allergic/atopic disease susceptibility and symptom severity [see reviews by (DunnGalvin et al., 2006; Pennell et al., 2012; Klein and Flanagan, 2016)]. However, male dominance of food allergy appears to be inconsistent across studies, depending on allergen types and patient age groups, as well as on the study method used and year examined (Jarvis and Burney, 1998; Becklake and Kauffmann, 1999; Kelly and Gangur, 2009; Acker et al., 2017). Therefore, the roles of these biological and immune dimorphisms in the sex-specific behavioral response to BLG sensitization and challenge are complex and require further scrutiny in humans as well as in our animal models of CMA.

Moving forward, we focused our pathophysiological investigation on male mice to assess histological and biochemical changes that might reflect the behavioral changes that deviated from the sham control. In orally-sensitized mice, the site of allergen insult is the gastrointestinal (GI) tract. Decreased mucosal occludin immunoreactivity and increased proinflammatory cytokine expression in the BLG-sensitized ileum suggested that the immune responses to the allergen during sensitization had impaired intestinal barrier (Figure 6). In addition, it is possible that dysbiosis had occurred during the sensitization and elicited these changes in intestinal physiology, since gut microbe compositions can be influenced by diet and shifts in compositions can result in

inflammation (Round and Mazmanian, 2009; Clements and Carding, 2018). These changes in the gut physiology and microbiota are likely to be produced gradually during the sensitization period, rather than immediately after the BLG challenge, since immune activation status in food-allergen sensitized mice has been reported to be heightened as evidenced by greater proliferative capacity of splenocytes compared to naïve mice without restimulation with the allergen (Li et al., 2000). However, time course of pathophysiology development and potential involvement of gut microbiota are yet to be determined in our mouse model. Loss of intestinal barrier and intestinal dysbiosis have been reported in autistic patients (de Magistris et al., 2010; Fiorentino et al., 2016) and their implication in pathogenesis of neuropsychiatric conditions has been reviewed in recent literature (Karakula-Juchnowicz et al., 2016; Grochowska et al., 2018).

Inflammatory responses were also found in the brain of BLG-sensitized mice. Although we did not detect apparent microgliosis by Iba1 immunostaining (not shown), we observed notable differences in GFAP-positive astrocyte morphology in certain areas of the brain, especially perivascular regions of the midbrain (Figure 7). Interestingly, similar observations of hypertrophic perivascular astrocytes have been observed in our WP-sensitized aged mice (Germundson et al., 2018) and also reported in the spinal cord of EAE mice (Voskuhl et al., 2009). These astrocytes resemble scar forming astrocytes often described in central nervous system injuries and are thought to establish barriers to control infiltration of leukocytes from the blood circulation (Voskuhl et al., 2009; Sofroniew and Vinters, 2010). Importantly, increased expression of GFAP plays a crucial role in this barrier formation since ablation of GFAP-expressing astrocytes results in profound increases in the number of leukocyte infiltrates in the spinal cord of EAE mice

(Voskuhl et al., 2009). Thus, it is feasible to postulate that BLG sensitization stimulated peripheral immune cells and increased their circulating levels, and the perivascular astrocytes had become activated to regulate the amount of inflammatory influence from the periphery. To provide evidence for this notion, juxtaposition of leukocytes with GFAP-immunoreactive astrocyte end-feet across the blood vessel walls need to be demonstrated in our mouse model of CMA as shown in the EAE mice (Voskuhl et al., 2009). Nonetheless, semi-quantitative analysis with western blotting showed that the GFAP levels in the midbrain regions were significantly elevated in BLG-sensitized mice when compared to sham mice (Figure 8), supporting our immunohistochemical observations.

Astrocytes are multifaceted glia cells in the central nervous system, and they play essential roles in metabolic support, intercellular signaling, blood flow regulation, myelination, and synaptic pruning [reviewed by (Sofroniew and Vinters, 2010)]. It is of interest to examine whether these functions of astrocytes become dysregulated in BLG-sensitized mice and influence their behavior. Astrocytes are also important mediators of neuroinflammation with the ability to produce and secrete pro- as well as anti-inflammatory molecules (Eddleston and Mucke, 1993; John et al., 2003). The fact that the levels of TNFα were significantly elevated in the midbrain regions of the BLG-sensitized mice suggested that the astrocytes were acting as proinflammatory mediators (Figure 10). However, it seems counterintuitive that microglia did not show reactive morphology in response to the elevated proinflammatory cytokine levels. One possible explanation is that TNFα detected in our samples had derived from the intestines or circulating leukocytes and was not produced by astrocytes, which had successfully

prevented the cytokine and cytokine-producing cells from activating microglia. An alternative explanation may be that our experimental paradigm was too transient, and BLG-sensitized mice needed to be repetitively challenged to elicit more chronic inflammation in order for microglia to become activated. These hypotheses, along with the possible involvement of other proinflammatory cytokines, such as IL-1β and IL-6, need to be tested in future studies.

In conclusion, we have demonstrated that sensitization of C57BL/6J mice with BLG induces anxiety- and depression-like behaviors in male mice that are associated with decreases in tight junction proteins in the intestines and astrogliosis in the brain. Elevated TNFα levels in both of these locations suggest that this proinflammatory cytokine plays a role, at least in part, in mediating immune responses to the cow's milk allergen in sensitized mice. Whether these pathophysiological findings directly influence the behavior of sensitized mice is yet to be determined. However, clinical reports of symptom improvements in patients with treatment-resistant depression and other psychiatric conditions after elimination diet (Parker and Watkins, 2002) and plasmapheresis (Barzman et al., 2018) support the involvement of FAH-triggered immune responses in pathogenesis of behavioral disorders. Treatments with antihistamines and/or steroidal/non-steroidal anti-inflammatory reagents to, respectively, inhibit the effects of hypersensitivity-mediated immediate immune reactions (e.g., mast cell degranulation) and subsequent inflammation in our mouse model will be useful in clarifying the involvement of proinflammatory cytokines in the development of observed brain pathophysiology and behavioral changes. Elucidating the mechanisms by which immune responses to a dietary component manifest as brain and behavioral dysfunction

may therefore provide potential therapeutic approaches beyond the use of neuromodulatory drugs.

Study 2 – Differential Myelination and Blood-Brain Barrier Associated Pathway Activation in Non-anaphylactic Model of Cow's Milk Allergy

Previous findings by us and others have characterized the behavioral phenotype arising from CMA (de Theije et al., 2014; Germundson et al., 2018; Smith et al., 2019; Smith et al., 2021). C57BL/6J mice are known to present mild symptoms compared to other mouse strains such as BALB/cJ and C3H mice which we observed here (Figure 11) (Smit et al., 2011; Marco-Martin et al., 2017; Smith et al., 2021). We have previously found no evidence of anaphylactic symptoms or allergen-induced hypothermia in C57BL/6J mice (Smith et al., 2021). Despite the lack of anaphylactic symptoms, they are routinely found to have elevated levels of allergen-specific IgE (Smit et al., 2011; Germundson et al., 2018; Smith et al., 2019; Smith et al., 2021). The mice generated for this study were treated similarly to those in our previous studies and have yielded consistent results (Figure 1); thus, behavioral testing was not performed with this group of animals.

Cytokines serve as likely mediators for peripheral to central signaling. BALB/cJ mice also presented with a robust behavioral phenotype and released large amounts of cytokines following allergen exposure (Marco-Martin et al., 2017; Smith et al., 2021). In addition, our previous observations with C57BL/6J mice implicated a TNFα mediated response causing astrogliosis and perivascular cuffing (Smith et al., 2019). Building off our CNS findings and cytokines profile seen in C57BL/6J mice following allergen challenge, we further validated those data by performing the same ELISA multiplex.

Despite not reaching the statistical significance threshold as previously observed with IL-10, IL-13, and other cytokines (Figure 12), similar trends were observed. To validate this observation, greater sample numbers will be required. Though our significant cytokine findings show little overlap with previously published work, the heterogeneity in our system may be the result of differences in cytokine release similar to what was observed when comparing C57BL/6J and BALB/cJ mice. Therefore, there may be evidence of functional overlap between our previous findings of increased chemokines and IL-10, Il-13, and IL-21 and roles of CCL24 and CXCL13 observed in this study.

Interestingly, despite evidence of gut barrier breakdown due to loss of occludin immunoreactivity (Smith et al., 2019), we observed no changes in the transcription of the tested factors or evidence of gut leakiness to FITC-dextran (Figure 13 and 14). The gut is the principal site of insult by CMA though we did not see evidence of permeability using this FITC-dextran approach, our model potentially does not yield a robust gut pathology or differing results may be achieved by adjusting our assay.

The midbrain has routinely been one of the areas we observed histological changes, including astrogliosis and mast cell degranulation (Germundson et al., 2018; Smith et al., 2019). Therefore, it was interesting that IPA predicted changes in FMR1 activity (Figure 15 and 18), decrease in myelin and lymphocyte protein (MAL) and proteolipid protein 1 (PLP1) transcription (Figure 18). The transcription of the iron transporter; transferrin was reduced. Transferrin is a central factor in the activity and differentiation of oligodendrocytes (Espinosa de los Monteros et al., 1999; Pérez et al., 2013; Marziali et al., 2016). When performing our pathway analysis in the midbrain region, a clear association to the ALS signaling pathway supported the potential for

myelin degradation. Interestingly, the perivascular cuffing of astrocytes seen in our previous work is well documented in EAE (Voskuhl et al., 2009; Sofroniew and Vinters, 2010). Regardless of the supportive association to myelin degradation pathways in IPA findings, we did not observe changes in myelination histologically when observed by MBP immunostaining, but there could be selective changes for other myelin proteins like the aforementioned MAL and PLP1. The iron transport dysfunction may have non-oligodendroglial effects, could take more time to cause visible effects due to transferrin's half-life of 8-10 days, or disruption of transferrin transcription does not impact already formed myelin.

Based upon pre-existing results in our model system that demonstrated perivascular astrocyte hypertrophy and TNFα accumulation in the midbrain, we suspected that blood-brain barrier integrity might be compromised (Smith et al., 2019). Evidence suggests that the brain is susceptible to the influx of immune cells and their products in inflammatory conditions (Rezai-Zadeh et al., 2009; Su and Federoff, 2014). Based on the observed reduction in *Ocln* transcription in our samples (Figure 19) and the predicted reduction in VEGFA in the pathway analysis (Figure 18), we assayed the permeability of the blood-brain barrier to serum IgG (Figure 21) (Hawkins and Davis, 2005; Hawkins et al., 2007; Argaw et al., 2012). High amounts of IgG in the brain parenchyma and other blood-brain barrier permeability markers have been following peripheral immune responses resulting from TNFα signaling or other mast cell products (Cheng et al., 2018; Tran et al., 2019; Shelestak et al., 2020; Huang et al., 2021). We did not expect permeability to IgG to the scale we observed; the blood-brain barrier pathology is likely a central component of the mechanism causing the behavioral change

and immune cell infiltration. These data will be further validated with our research going forward as we continue to unveil the peripheral to central signaling cascade linking CMA to anxiety behavior.

We were not able to investigate all the identified pathways in-depth, though they may serve as a foundation for future studies. The FcεRI had a positive z-score in the midbrain, which corresponds with our previously published studies showing mast cell degranulation. Though this project's focus was cytokine signaling, other mast cell factors are likely released by degranulation upon FcεRI activation. Across the midbrain and striatum/frontal cortex, signaling pathways involved in synaptogenesis, neurite development, axonic development, and netrin signaling were differentially activated. Changes in the activity of axonal, neurite, and synaptic development pathways all imply potential changes in neuronal morphology and connectivity. Neuronal morphology was outside the focus of this study but may be confirmed using general or selective neuronal immunohistochemical stain combined with a 3D structural scoring like a Sholl analysis (Sholl, 1953; Binley et al., 2014). Though histamine functions as a neurotransmitter, did not detect prediction of histamine signaling changes in our pathway analysis. Due to the crude dissection method used in our study, small transcriptional changes in confined regions might not have been detected with a great resolution. Because all histaminergic neurons are located in the tuberomammillary nucleus of the hypothalamus, transcriptional changes within this small nucleus might have been diluted by the inclusion of surrounding tissue (Scammell et al., 2019). In addition, the postsynaptic cells of the tuberomammillary nucleus are broadly scattered throughout the forebrain in the striatum, preoptic area, and prefrontal cortex; therefore, a high-resolution analysis may be required.

Despite the lack of detection for differences in histaminergic signaling, some CMA-associated neuronal signaling pathways were identified by IPA. Most notably, the endocannabinoid and opioid signaling pathways have negative and positive activation z-scores, respectively. Interestingly general G-protein coupled receptor subunit αs was increased in the striatum and frontal cortex sample. These signaling pathways warrant further investigation as they have document impacts on behavior.

In conclusion, we provided further support for a mechanism by which a non-anaphylactic mouse model of CMA might elicit anxiety- and depression-like behavioral changes. BLG-sensitized mice were confirmed allergic by induction of BLG-specific IgE and lacked any apparent anaphylactic symptoms or health impact. The profile of plasma cytokines had considerable overlap with the profile previously observed in male C57BL/6J mice. The significantly increased BLG mouse cytokines and chemokines IL-10, IL-13, CCL24, and CXCL13. Significant changes in brain transcriptome and histopathology were also observed. Our analysis suggested that Fmr1, Dio2, Slc16a2, and Sox2 likely drive the relevant pathways for the neuro and behavioral pathologies observed. In the midbrain, changes in myelination, anxiety, FcεRI, and vascular permeability were also predicted. These changes are likely important for the observed behavioral alterations in our BLG-sensitized mice.

Study 3 – Anxiety-like Behavior and Intestinal Microbiota Changes as Strain- and Sex dependent Sequelae of Mild Food Allergy in Mouse Models of Cow's Milk Allergy

The severity and presentations of food allergy symptoms widely vary among individuals and likely contribute to inconsistencies across human cohort studies that investigate the association of food allergy with affective, behavioral, and cognitive disorders. Indeed, the heterogeneity in responses to allergens is often observed in human patients (Burks et al., 2012; Bird et al., 2015; Sicherer and Sampson, 2018; Fritscher-Ravens et al., 2019). We postulated that the presence of allergy-associated behavioral manifestations would be influenced by sex and genetic background. In this study, we therefore used a mouse model of CMA to evaluate the effect of strain and sex on CMA-associated anxiety-like behavior and cognitive function as well as physical reactions, immunological responses, and microbial changes.

We first assessed whether the BLG sensitization regimen effectively induced CMA. Following an oral allergen challenge, both C57BL/6J and BALB/cJ strain mice showed no or mild observable reactions, scoring a severity level of 0-2 upon allergen challenge (Figure 22B). This outcome was not unexpected given that we and others have previously reported mild responses to allergen challenges with these strains (Marco-Martin et al., 2017; Germundson et al., 2018; Smith et al., 2019), and C57BL/6 and BALB/c backgrounds are known to be more resilient to experimental allergic sensitization than other strains (Xu et al., 2018). However, greater numbers of BLG-sensitized BALB/cJ mice scored 2 than sex- and treatment-matched C57BL/6J mice, rendering the modest differences in the clinical scores and body temperature from their respective sham groups statistically significant (Figure 22B, C). Mast cell-derived

histamine is a known contributor of allergy-induced hypothermia and respiratory distress, acting via H1/H2 histaminergic receptors. The absence of mast cells or histamine production in knockout mice, as well as histaminergic receptor antagonists in wild-type mice, have been shown to ameliorate these symptoms after inducing passive systemic anaphylaxis (Makabe-Kobayashi et al., 2002). Thus, our observation suggested that BLG-sensitized BALB/cJ mice were more susceptible to mast cell degranulation upon allergen challenge. As for the allergen-specific immunoglobulins, the sensitization-induced changes in their levels did not closely mirror the physiological responses, and all BLG groups, except C57BL/6J females, showed small but significant increases in allergen-specific IgE (Figure 23A). Furthermore, IgG isotypes were also elevated in both male and female BALB/cJ mice after sensitization (Figure 23B and 23C). In contrast, we did not observe elevated IgG1 in the BLG groups of C57BL/6J mice. These results indicated that the production of allergen-specific immunoglobulin was differentially affected by the strain, particularly highlighting the difference in the amounts of IgG1 production. However, we have previously observed elevated BLG-specific IgG1 in male and female C57BL/6J mice in our earlier study, in which mice were challenged twice, 1 week apart (Smith et al., 2019). Thus, the number of allergen exposure and/or duration after sensitization may also affect the amounts of allergen-specific immunoglobulins.

Despite the lack of anaphylaxis and other overt physical indications of severe allergic responses, BLG-sensitized C57BL/6J mice exhibited anxiety-like behavior one day after the allergen challenge. This CMA-associated behavior change was not observed in female C57BL/6J mice, an outcome in line with our previous study (Smith et al., 2019). Although the results from the OF test were difficult to compare in BALB/cJ mice

due to their overall inactivity, the differences between sham and BLG mice were detected for male mice by the number of fecal pellets and EZM (Figure 24D and 25A-C). Again, these differences were not observed in BALB/cJ female mice, suggesting that females were less inclined to exhibit CMA-associated anxiety-like behavior regardless of strain. The cross-maze test did not show significant differences between sham and BLG mice in any of the groups or strain- or sex-dependent effects, suggesting that BLG sensitization followed by acute exposure to the allergen did not affect cognitive function, at least for spatial memory (Figure 26).

Similar male-biased symptom manifestations have also been reported in human patients. Food allergy and certain types of behavioral conditions, such as ADHD and autism, are more commonly diagnosed in young males (Polanczyk et al., 2007; Kim et al., 2011; Pinares-Garcia et al., 2018). The sex-dependent dichotomy may be explained by the differences in the number of T lymphocytes and the presence of promoter elements within immune-related genes that can be regulated by sex hormones (DunnGalvin et al., 2006; Markle and Fish, 2014; Klein and Flanagan, 2016; Laffont and Guery, 2019). In rodent studies, the contribution of estrogen to the regulation of anxiety-like behavior has also been investigated, although anxiolytic effects seem to depend on the dose of the hormone, behavior tests used, and age of mice (HayGlass et al., 2005; Boivin et al., 2017; Borrow and Handa, 2017). While it was outside the scope of our current study, identification of mechanistic or molecular factor(s) that protected our female mice from manifesting CMA induced anxiety-like behavior may provide potential therapeutic targets for anxiety.

Sex- and strain-specific variations were also found in the number, type, and levels of immune mediators detected in plasma, with male C57BL/6J mice showing the most number of analytes that were significantly altered with BLG sensitization, followed by male and female BALB/cJ mice (Figure 27A-C). No significant changes in plasma mediator levels were detected in female C57BL/6J mice, again underscoring the resilience of this group to the sensitization. In C57BL/6J males, increases in Th cells associated cytokines and chemokines were particularly notable. While the C57BL/6 and BALB/c strains are typically described to have Th1- and Th2- biased immune responses, respectively (Autenrieth et al., 1994; Nishimura et al., 1997; Mills et al., 2000; Watanabe et al., 2004), it has been argued that this generalization is based on infection models and does not apply to allergy paradigms (HayGlass et al., 2005). Our results also seemed to support this argument and did not categorize the responses of the two strains strictly into either Th1 or Th2. Instead, significant increases in IL-13 and IL-21 suggested that the responses of BLG-sensitized C57BL/6J mice were likely mediated by follicular helper T cells (Tfh), which are crucial for the production of IgE and other isotypes via differentiation of B cells (Gowthaman et al., 2019; Yao et al., 2020).

The mediator responses by male C57BL/6J and BALB/cJ mice were clearly contrasting, with the former males showing increases in some mediators and the latter exhibiting decreases in a distinct set of mediators. In particular, sensitization-associated changes in IL-13 were observed in both strains but in opposite directions (Figure 27A, B). Although the functional significance of these CMA-induced differential changes in the analytes is yet to be determined, the elevation of IL-13 in C57BL/6J males likely facilitated the production of BLG-specific IgE. Concurrent increases in IL-10,

FGF2, CSF1, and CCL chemokines in C57BL/6J males also suggested that mobilization and/or proliferation of immune cells were prompted, triggering complex systemic pro- and anti-inflammatory responses in BLG-sensitized mice of this strain. It should be noted that some of these cytokines had been reported to be elevated in mouse models with more severe allergic reactions. For example, CCL1 and CCL17 were induced in the intestines of ovalbumin (OVA)-sensitized mice that developed diarrhea with mast cell infiltration (Knight et al., 2007), and high levels of CCL9 (MIP-1γ) was found in OVA-sensitized mouse lungs with increased airway resistance and eosinophil infiltrates (Rose et al., 2010). These results suggested that, despite their lack of overt allergic reactions, BLG-sensitized C57BL/6J mice elicited similar immune responses to those of other animal models of severe allergies, and thus, the absence of typical allergic reactions might not necessarily indicate the absence of hypersensitivity to the allergen. Following the same line of argument, the changes in the plasma mediators may not be accurate indicators of CMA-associated behavioral manifestations. As mentioned above, the cytokine profile of BALB/cJ male mice was conflicting with their C57BL/6J counterpart while both groups displayed anxiety-like behavior when assessed with the EZM test. In addition, a few of the inflammatory mediators were elevated in BALB/cJ female mice after sensitization (Figure 27C), but this group did not exhibit behavioral abnormalities, at least with the tests we performed.

It may also be argued that the observed cytokine changes were reflective of different stress levels mice might have experienced from the behavior test one day before their blood was collected. Indeed, it has been shown that foot-shock stress increases the amounts of inflammatory cytokines in mice, including IL-10 and IL-13 (Cheng et al.,

2015). However, the plasma levels of these cytokines reportedly return to the control levels after 24 hrs (Cheng et al., 2015), rendering the possibility that stress was a major contributor of the cytokine/chemokine changes less likely. Taken together, testing for the altered plasma levels of immune mediators may be more sensitive in detecting the development of allergen hypersensitivity than the presence of physical reactions, although it may not predict the presence of allergen-induced behavioral abnormality.

We then postulated that intestinal microbiota would be altered following BLG sensitization procedure. Altered microbiota, or dysbiosis, has been reported in individuals with food allergy as well as with neuropsychiatric disorders (Tomova et al., 2015; Aizawa et al., 2016; Bunyavanich et al., 2016; Mangiola et al., 2016; Cenit et al., 2017; Savage et al., 2018). In addition, a growing amount of evidence supports that microbiota influences behavior and mood (Tomova et al., 2015; Aizawa et al., 2016; Mangiola et al., 2016). Indeed, we found clear differences in beta diversity at the phylum level between sham and BLG-sensitized mice in all groups, although the most notable change with BLG sensitization was a marked decrease in Verrucomicrobia (Figure 28 and 29). While the number of observed species remained relatively unchanged (Figure 28A), Simpson index for C57BL/6J male mice indicated decreased biodiversity, supporting previous findings in humans that changes in alpha diversity was associated with milk allergy (Berni Canani et al., 2018; Shen et al., 2019). We further demonstrated that *A. muciniphila* was significantly lowered in the BLG-sensitized male C57BL/6J mice while its amount was increased in sham mice during the course of sensitization (Figure 30). This observation suggested that the colonization of the bacteria in the intestines was restricted during allergy development. *A. muciniphila* has been reported to be an important commensal

inhabitant that protects epithelial barrier integrity by regulating mucus production in the host (Reunanen et al., 2015). Furthermore, altered relative abundance of *A. muciniphila* has been found in obesity, diabetes, inflammatory bowel disease as well as neurological diseases such as Parkinson's disease, and autism (Wang et al., 2011; Everard et al., 2013; Hill-Burns et al., 2017; Li et al., 2019; Xu et al., 2019). Thus, it is possible that the lack of *A. muciniphila* is associated with anxiety-like behavior and/or other behavioral manifestations. Further studies in which the amounts of intestinal *A. muciniphila* are experimentally manipulated prior to behavior testing will confirm this association. Our KEGG Pathway analysis further supported that the microbiome changes observed in BLG-sensitized male C57BL/6J mice likely influenced neurological functions, including those involved in Parkinson's disease and dopaminergic and serotonergic synapses (Figure 31). These pathways consist of many molecular interactions between enzymes, receptors, channels, transporters, etc., and therefore it is necessary to validate the involvement of specific pathway components that were indeed affected by the sensitization-induced dysbiosis. This extrapolated bioinformatics approach, however, provides valuable information in narrowing down potential targets for further investigation in our future studies.

Taken together, our results demonstrated some significant sex- and strain-dependent differences in the symptom presentations of experimentally induced CMA in mice, with males of both strains having the propensity to display anxiety-like behavior. Other sensitization-associated differential presentations included serum levels of allergen-specific IgE/IgG, plasma levels of immune mediators, and changes in microbiota compositions. We hereby provide evidence that the manifestations of hypersensitivity to

the same allergen are influenced by genetic variables in individuals, and therefore diagnosing food allergy by immediate reactions after allergen challenge and immunoglobulin levels may exclude a population of individuals with milder or atypical responses. In addition, stratification of allergic cohorts with additional diagnostic criteria may reduce apparent inconsistencies in human studies.

Limitations of Work Presented in this Dissertation

The research presented in this dissertation highlights the causal role of CMA and various behavioral observations in a non-anaphylactic mouse model. Limitations of the work discussed in these studies are presented as follows.

These data were generated in selective wild-type backgrounds of the C57BL/6J and BALB/cJ strains. These strains, as previously discussed, were chosen due to their archetypal nature of Th1 and Th2 immune biases (Autenrieth et al., 1994; Nishimura et al., 1997; Mills et al., 2000; Watanabe et al., 2004). Though the use of these inbred strains of mice removed the inherent genetic variability that has likely contributed to the conflicting results seen in human studies, applying these findings to heterogeneous populations is still necessary. However, the consistencies across the two distinct mouse backgrounds is a promising preliminary finding that may be further built upon using outbred mouse colonies.

The measurement of allergen-specific IgE in the circulation is that it may not precisely reflect the total amount of the IgE produced by an allergic individual, since produced IgE becomes rapidly associated with high-affinity FcεRI on immune cells such as mast cells and basophils, and free IgE molecules are subjected to rapid degradation (Lawrence et al., 2017). Moreover, the serum samples we used in this study were prepared from the terminal blood, which was collected after 1 week from the last sensitization dose and 1-2 days from the allergen challenge (see Figure 1A). The half-life of IgE is estimated to be 2-3 days in humans (Lawrence et al., 2017) and 12 hours in mice (Vieira and Rajewsky, 1988), and thus, the amounts of IgE detected in our assay may have been an underestimation of actual amounts produced. Another limitation of our

current study was that IgG2a was measured as one of the allergen-specific immunoglobulins. However, C57BL/6J mice do not produce IgG2a but instead produce IgG2c isotype (Jouvin-Marche et al., 1989). For further assessments for allergen-induced immunoglobulin levels in this strain, an IgG2c-specific assay should be used.

Despite evidence of gut barrier breakdown previously alluded to by loss of occludin immunoreactivity, we observed no changes in the transcription of the tested factors or evidence of gut leakiness to FITC-dextran. The gut is the principal site of insult for CMA, so the lack of a robust pathology is concerning; however, altering our approach may yield differing results. For example, we collected serum 5 hours after feeding FITC-dextran, when some protocols have reported results with shorter collections (Woting and Blaut, 2018; Bordoni et al., 2019). Therefore, our mice might have cleared the FITC-dextran before it was quantitated, which could also explain the high levels of variability of the FITC signal.

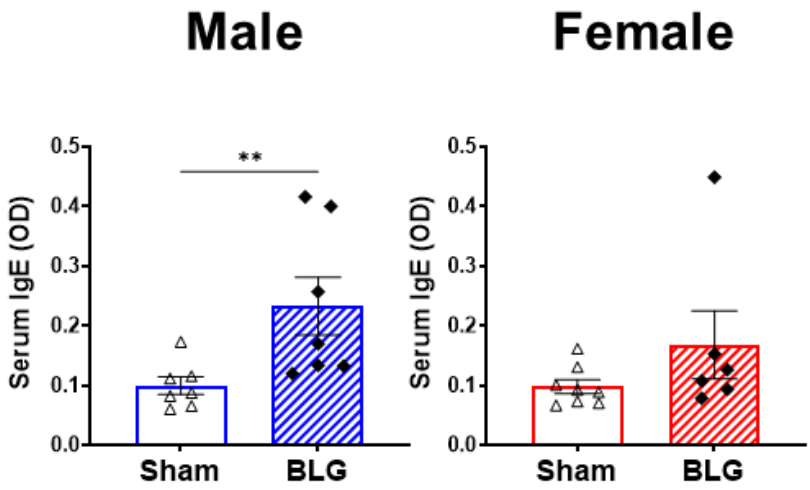
In our analysis of the brain, we broadly sectioned it into 4 regions. Despite the source of our RNA being from large heterogeneous regions of the brain containing many cell types and function regions, we still found differentially expressed genes. More genes may have been identified if we took a more directed approach or isolated specific cell types. For example, innovations in single-cell sequencing technologies, including Visium spatial gene expression, have seen great success and would allow for profiling RNA expression and mapping back onto histological sections (Maniatis et al., 2019; Mantri et al., 2021). Regardless a large number of regional differentially expressed genes were identified. To streamline our analysis, we performed pathway analysis and focused on changes of interest.

Summary of Conclusions and Future Directions

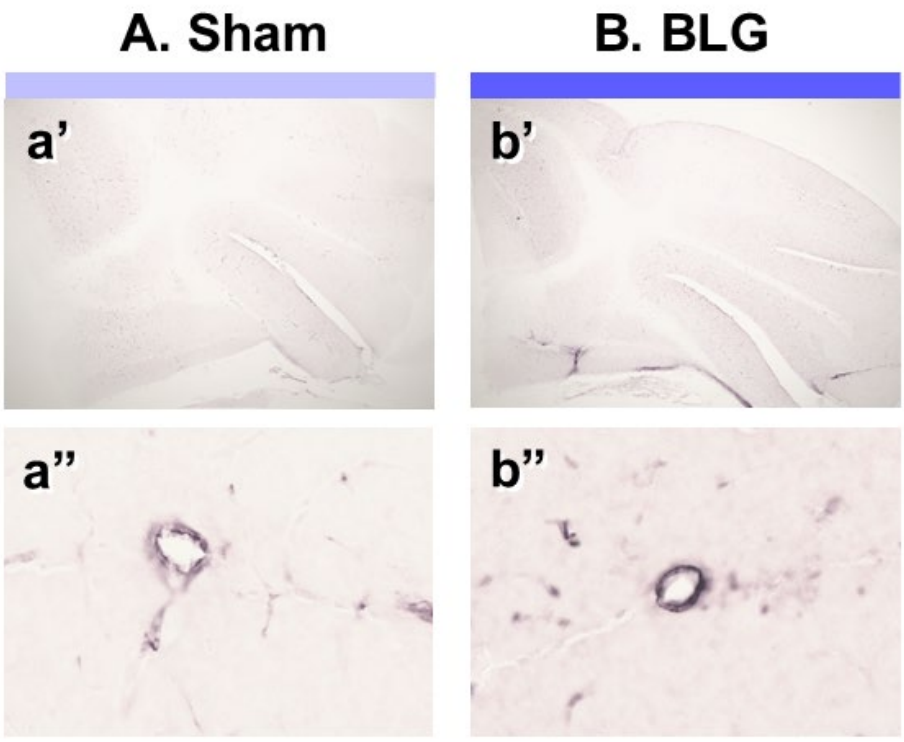
The work presented in these studies characterize the systemic response resulting from non-anaphylactic CMA sensitized to the milk allergen BLG. BLG allergy within our model elicits varied responses depending on genetic background and sex. Universally in mice that exhibited anxiety- and depression-like behaviors, BLG-specific IgE was elevated. The intestinal impact of BLG allergy impacted gut health, causing dysbiosis. Breakdown on normal gut microenvironment likely exacerbated both the peripheral immune response and promoting behavioral changes. From the immune system's provocation, elevated circulation of various cytokines further promoted immune activity and likely acted as peripheral to central signaling molecules. Within the central nervous system, transcriptional changes for various signaling and overall health pathways were altered. Most interestingly, evidence of the breakdown of tight junctions and cellular function of the blood-brain barrier were noted. These findings were validated by prominent perivascular astrocyte hypertrophy and build-up of extravascular IgG in the midbrain, likely resulting or involved with the increase in TNFα release.

Future work in this research area will continue fleshing out the molecular mechanism taking place within the brain. This will include further investigation of the exact role of mast cells, further characterizing the cellular morphology and signaling evidence established in this dissertation. We will also explore intervention strategies in the form of gut microbiota support with probiotics, mast cell suppressive mechanism, and interruption of peripheral to central signaling mechanisms through TNFα, for example.

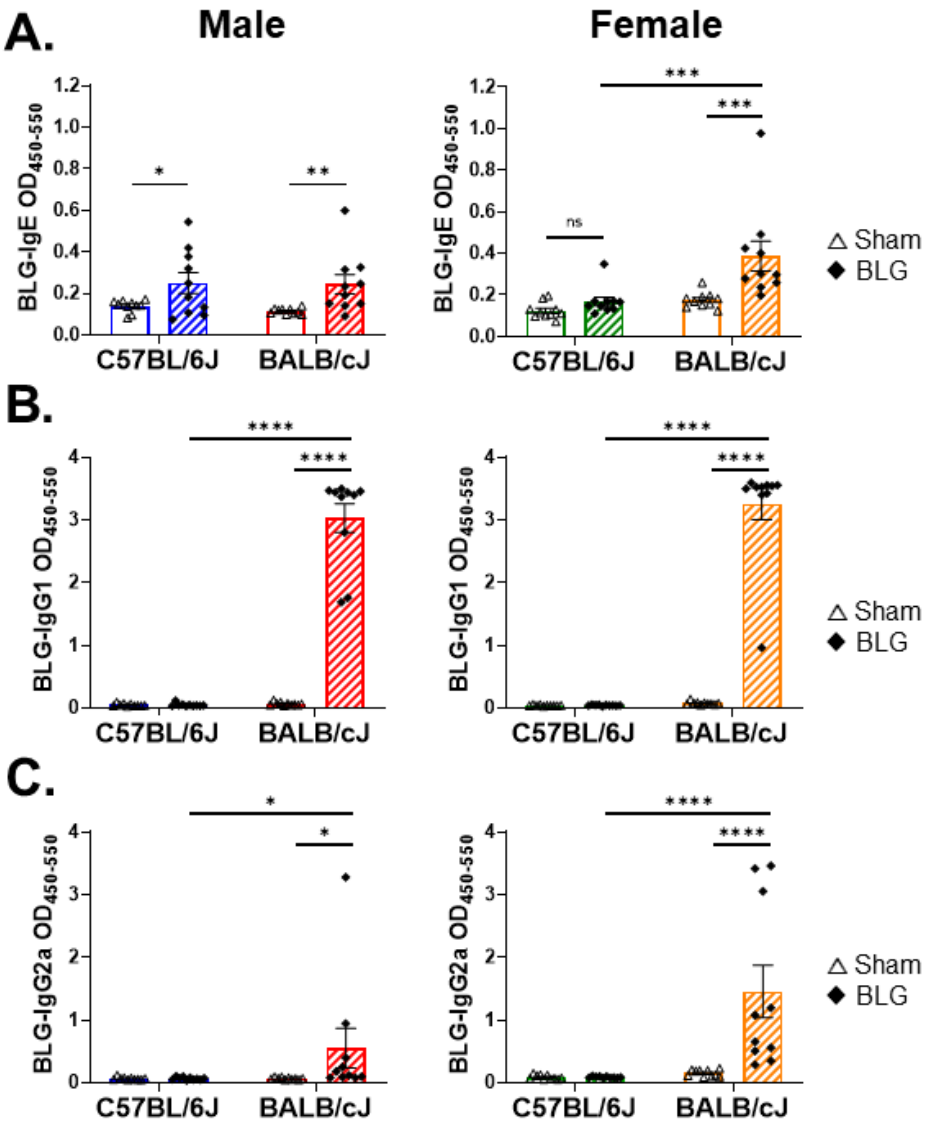
SUPPLEMENTAL FIGURES



Supplemental Figure 1. An alternative analysis for the post-sensitization serum levels of BLG-specific IgE shown in Figure 4B. Serum isolated from the terminal blood was used to quantify the levels of BLG-specific IgE using ELISA. A group analysis including all sample values are shown in Figure 2B. As an alternative analysis of the results, outliers within each group were identified using GraphPad Prism software (ROUT, Q = 1%), and Mann-Whitney test was performed excluding the outlier values from the statistical analysis. For male groups, statistical significance of $^{**}p < 0.01$ was found between sham and BLG mice (male sham: 0.10 ± 0.02, $n = 7$; male BLG: 0.23 ± 0.05, $n = 7$; one outlier from each group was removed from the analysis [sham, 0.40; BLG, 2.37]). Statistically significant difference between female sham and BLG groups was not found using this method of analysis [female sham: 0.10 ± 0.01, $n = 8$; female BLG: 0.17 ± 0.06, $n = 6$; two outliers removed from the analysis of the BLG group [3.58, 2.32].

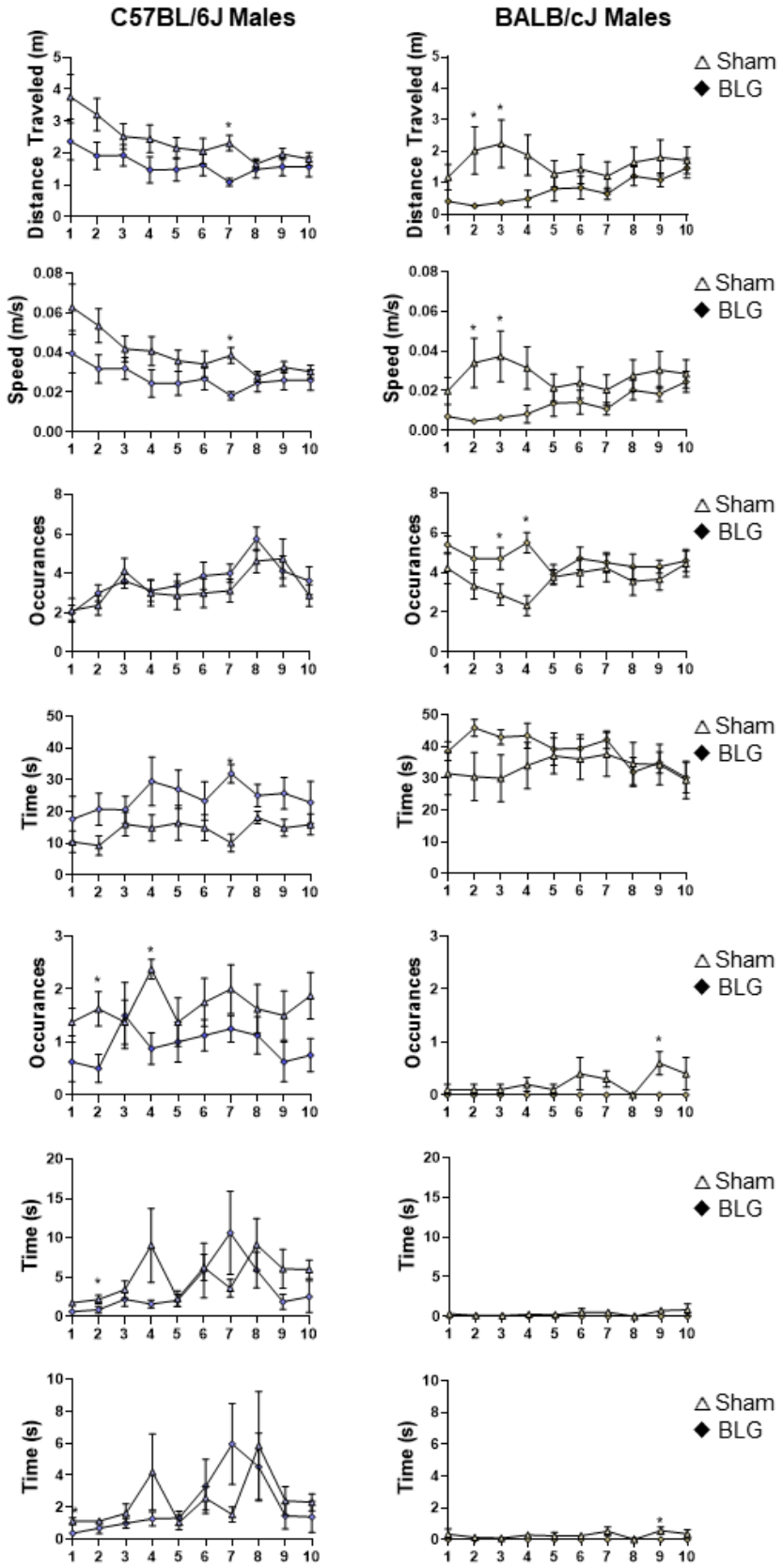


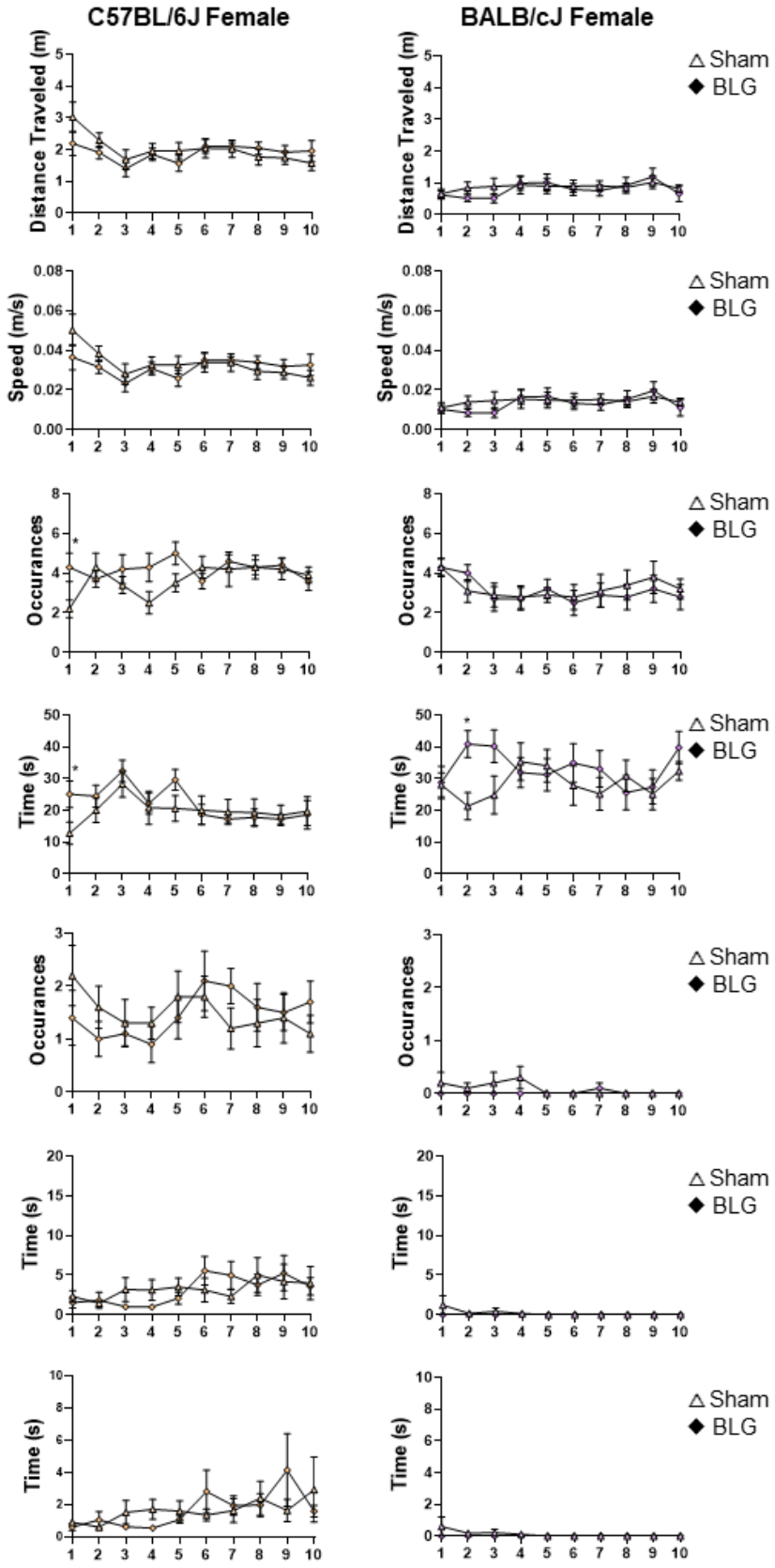
Supplemental Figure 2. Additional representative images of IgG immunoreactivity. The OD of IgG quantified extravascular IgG within the brain parenchyma (40 μm). Representative midbrain sections from sham (**A**, **a'**, **a"**) and BLG-sensitized (**B**, **b'**, **b"**) male mice are shown. Sections of the cerebellum (**a'**, **b'**) show areas where no difference in staining was observed across layers. Capillaries in cross-section adjacent to the hippocampus (**a"**, **b"**) were observed to have differential staining along the wall of the blood vessel wall. The cerebellum sections (**a'**, **b'**) and capillary cross-section (**a"**, **b"**) images taken with 4X and 40X objectives, respectively. Scale bars: 1 mm for (**A**) and (**B**); 50 μm for **a'-b"**.

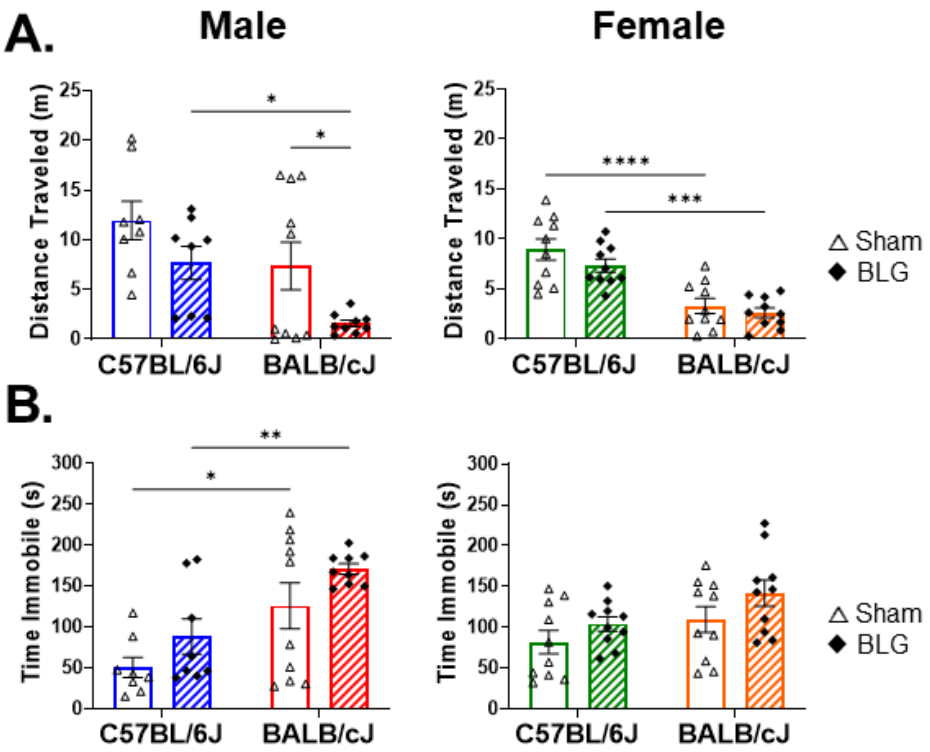


Supplemental Figure 3. Serum levels of BLG-specific immunoglobulin isotypes. Terminal blood samples were used to detect BLG-specific serum IgE (**A**), IgG1 (**B**) and IgG2a (**C**) using ELISA. Optical density values at 450 nm were used to plot the graphs after subtracting the background values at 550 nm (OD450-550). Sham mice (open bars with open triangles); BLG mice (striped bars with filled diamonds); male C57BL/6J (blue); male BALB/cJ (red); female C57BL/6J (green); female BALB/cJ (orange). Bars indicate group average values in OD450-550 ± SEM (two-way ANOVA), *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, $n = 10$.

Supplemental Figure 4. Time-dependent changes in the parameter measurements in the open-field activity monitoring. Overall activities of male (**S4A**) and female (**S4B**) sham and BLG-sensitized mice in an open-field arena were recorded for 10 min, and the activity parameters indicated in the y-axes were quantified by ANY-maze software. The values were graphed in relation to time in minutes (x-axis) during the test duration. Sham: open triangles; BLG-sensitized: filled diamonds. Values indicate group average ± SEM (t-test), $*p < 0.05$, $n = 8\text{-}10$.


C57BL/6J Males
BALB/cJ Males
Distance Traveled (m)
Speed (m/s)
Occurances
Time (s)
Occurances
Time (s)
Time (s)
△ Sham
◆ BLG

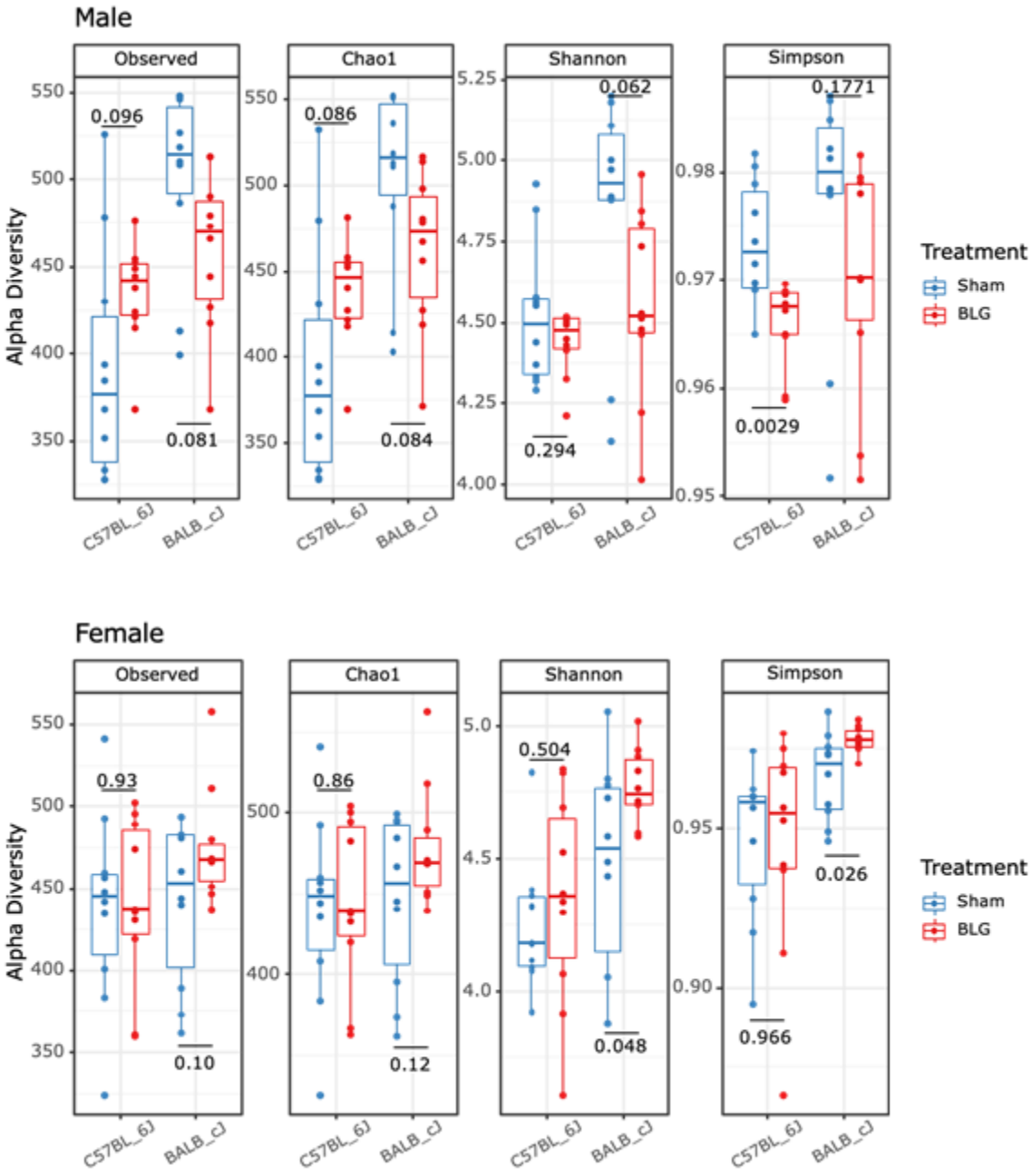

C57BL/6J Female
BALB/cJ Female
Distance Traveled (m)
Speed (m/s)
Occurances
Time (s)
Occurances
Time (s)
Time (s)
△ Sham
◆ BLG
*



Supplemental Figure 5. General locomotor activities recorded during the open-field activity monitoring. Total distance traveled and total time immobile were computed using ANY-maze software to assess potential effects of BLG sensitization on overall activities. Sham mice (open bars with open triangles); BLG mice (striped bars with filled diamonds); male C57BL/6J (blue); male BALB/cJ (red); female C57BL/6J (green) female BALB/cJ (orange). Bars indicate group average values ± SEM (two-way ANOVA), *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, n = 8-10.

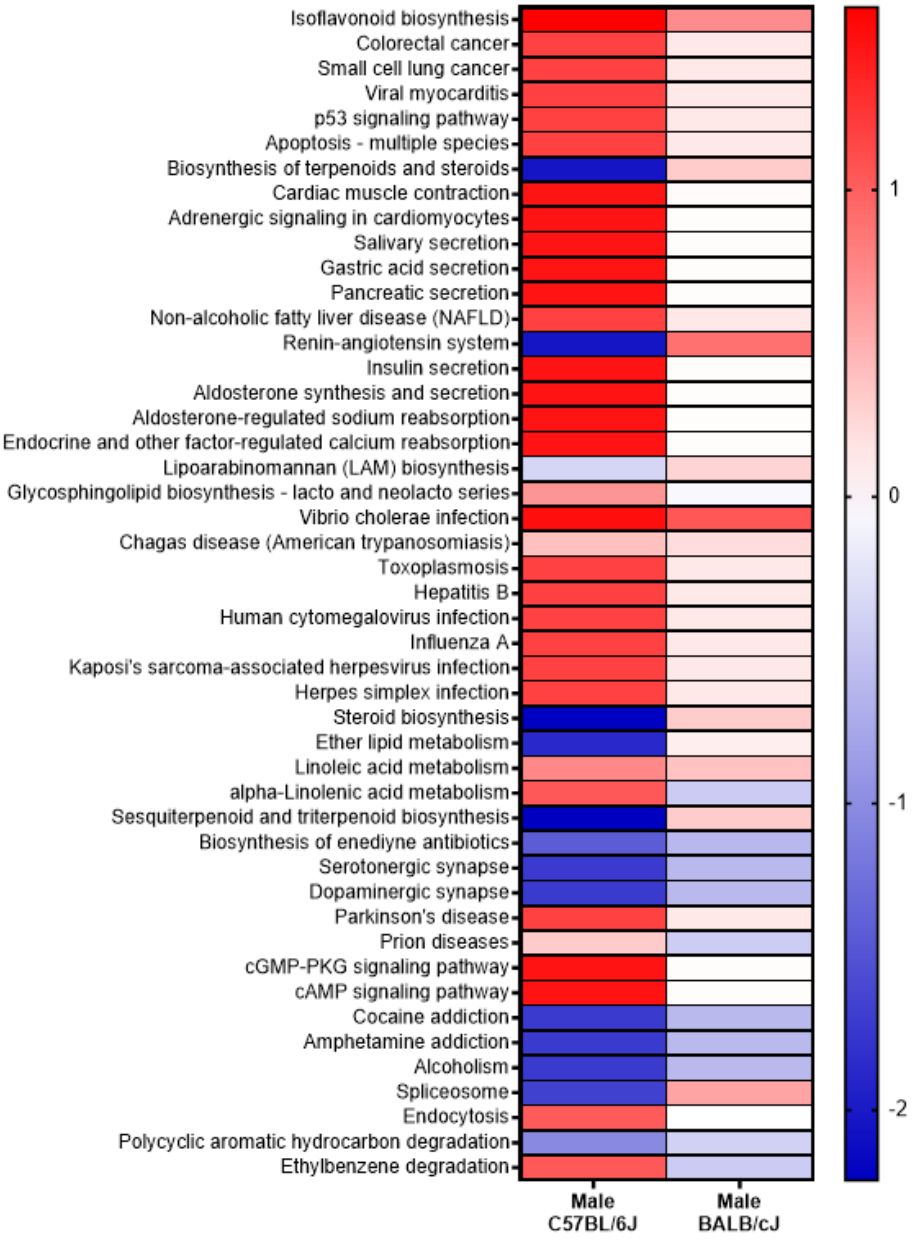
	Male C57BL6J				Male BALB/cJ			
Analyte	Sham Mean	BLG Mean	Fold change	p-value	Sham Mean	BLG Mean	Fold change	p-value
FGFβ	49.11 ± 11.02	195.02 ± 29.06	3.97	0.0001	1020.80 ± 143.11	735.46 ± 88.57	0.72	0.1655
CXCL13	1326.27 ± 489.02	1155.79 ± 252.84	0.87	0.9118	604.72 ± 93.99	510.88 ± 80.66	0.84	0.2475
CD30L	100.34 ± 34.57	219.55 ± 49.37	2.19	0.063	10.32 ± 3.54	0.59 ± 0.30	0.06	0.1462
CCL11	1050.32 ± 107.32	1069.74 ± 125.06	1.02	>0.9999	2290.56 ± 186.80	2218.84 ± 138.97	0.97	0.5787
CCL24	311.14 ± 123.35	344.08 ± 76.46	1.11	0.393	301.00 ± 41.04	348.44 ± 89.29	1.16	0.7394
FasL	1930.06 ± 635.53	964.37 ± 246.35	0.5	0.2475	137.78 ± 82.05	12.18 ± 6.24	0.09	0.0064
CSF3	27167.30 ± 15014.58	19855.08 ± 6673.43	0.73	0.9705	3807.01 ± 3136.01	651.39 ± 261.17	0.17	0.5149
CSF2	1889.84 ± 651.33	4012.78 ± 1236.58	2.12	0.0892	157.36 ± 25.05	49.12 ± 20.31	0.31	0.0021
ICAM-1	654.51 ± 269.44	1173.07 ± 274.22	1.79	0.1655	683.33 ± 332.29	320.19 ± 123.14	0.47	0.5288
IFNγ	2581.15 ± 1024.82	2146.25 ± 566.21	0.83	0.8534	1258.68 ± 646.21	355.33 ± 56.09	0.28	0.0355
IL-1α	21.65 ± 9.60	31.88 ± 8.18	1.47	0.2176	12.88 ± 4.23	10.88 ± 4.16	0.84	0.9118
IL-1β	259.10 ± 46.75	478.09 ± 151.25	1.85	0.4359	102.79 ± 15.02	55.73 ± 11.44	0.54	0.0232
IL-2	5515.71 ± 2935.97	17325.97 ± 9220.25	3.14	0.123	1178.89 ± 369.73	469.67 ± 138.26	0.4	0.1431
IL-3	1333.24 ± 621.26	1921.58 ± 596.36	1.44	0.4813	709.52 ± 558.68	82.28 ± 17.22	0.12	0.063
IL-4	0.00 ± 0.00	0.00 ± 0.00	3.93	0.393	0.00 ± 0.00	0.00 ± 0.00	0.13	0.1655
IL-5	5323.92 ± 2794.36	4985.74 ± 1345.51	0.94	0.4813	1194.88 ± 719.80	258.09 ± 71.19	0.22	0.0892
IL-6	429.68 ± 140.59	517.23 ± 137.71	1.2	0.5787	232.02 ± 93.17	89.55 ± 10.05	0.39	0.0753
IL-7	19.79 ± 6.18	32.02 ± 6.19	1.62	0.1648	61.96 ± 31.53	20.10 ± 7.72	0.32	0.0345
IL-10	177.08 ± 30.97	317.87 ± 52.30	1.8	0.0433	492.71 ± 196.54	231.89 ± 45.29	0.47	0.2799
IL-12p40	126.51 ± 114.36	110.98 ± 91.89	0.88	0.7959	16.16 ± 10.17	3.29 ± 0.92	0.2	0.1431
IL-13	779.74 ± 261.40	5353.06 ± 1667.05	6.87	0.0007	413.89 ± 58.89	215.54 ± 71.69	0.52	0.0288
IL-15	15044.49 ± 5557.76	27251.54 ± 6717.00	1.81	0.0892	982.38 ± 256.13	824.38 ± 214.97	0.84	0.8534
IL-17	501.59 ± 173.16	416.09 ± 96.73	0.83	0.9705	199.60 ± 153.99	34.87 ± 8.99	0.17	0.123
IL-21	41.38 ± 23.03	187.49 ± 32.63	4.53	0.0009	111.32 ± 61.74	51.77 ± 26.99	0.47	0.3815
CXCL1	100.27 ± 22.84	185.93 ± 51.58	1.85	0.0892	142.13 ± 17.81	127.99 ± 14.03	0.9	0.8534
Leptin	59.83 ± 28.40	87.74 ± 31.17	1.47	0.393	43.58 ± 18.61	16.32 ± 3.83	0.37	0.315
CXCL5	3497.46 ± 2707.21	7582.71 ± 2419.04	2.17	0.1655	4710.91 ± 1542.50	9041.47 ± 4266.91	1.92	0.8534
CCL2	516.44 ± 268.78	682.61 ± 243.27	1.32	0.6305	19.65 ± 9.33	5.05 ± 1.62	0.26	0.6842
CCL12	85.78 ± 35.08	257.50 ± 56.89	3	0.0185	86.40 ± 22.09	86.58 ± 16.12	1	>0.9999
CSF1	18.86 ± 9.56	176.38 ± 38.10	9.35	0.0001	49.49 ± 11.58	96.15 ± 31.55	1.94	0.4813
CXCL9	114.09 ± 36.86	74.19 ± 20.73	0.65	0.2799	84.52 ± 26.48	50.41 ± 12.56	0.6	0.4359
CCL3	790.33 ± 145.31	1276.22 ± 187.93	1.61	0.0753	303.61 ± 38.92	300.72 ± 61.46	0.99	0.8534
CCL9	3762.31 ± 355.11	4909.70 ± 191.10	1.3	0.0015	3814.88 ± 210.18	3512.86 ± 232.41	0.92	0.3527
PF-4	2322534.62 ± 222353.54	2596173.20 ± 161134.51	1.12	0.3527	1920479.48 ± 175611.44	1710952.26 ± 196698.41	0.89	0.6305
CCL5	754.18 ± 205.99	517.96 ± 158.22	0.69	0.6305	136.58 ± 103.16	25.16 ± 6.19	0.18	0.7959
CCL17	82.92 ± 21.96	527.57 ± 186.94	6.36	0.0039	67.17 ± 16.06	64.32 ± 17.93	0.96	0.6842
CCL1	1.08 ± 0.46	12.69 ± 4.16	11.78	0.0003	6.36 ± 2.80	6.91 ± 3.96	1.09	0.5149
TNFRI	1152.31 ± 114.96	1403.22 ± 133.45	1.22	0.063	1087.49 ± 121.09	565.51 ± 56.03	0.52	0.0002
TNFRII	9593.01 ± 6359.09	966.49 ± 348.09	0.1	0.9118	133.94 ± 43.12	220.89 ± 95.10	1.65	0.7394
TNFα	29.06 ± 9.10	49.27 ± 10.46	1.7	0.1224	77.18 ± 13.74	63.63 ± 13.46	0.82	0.4043

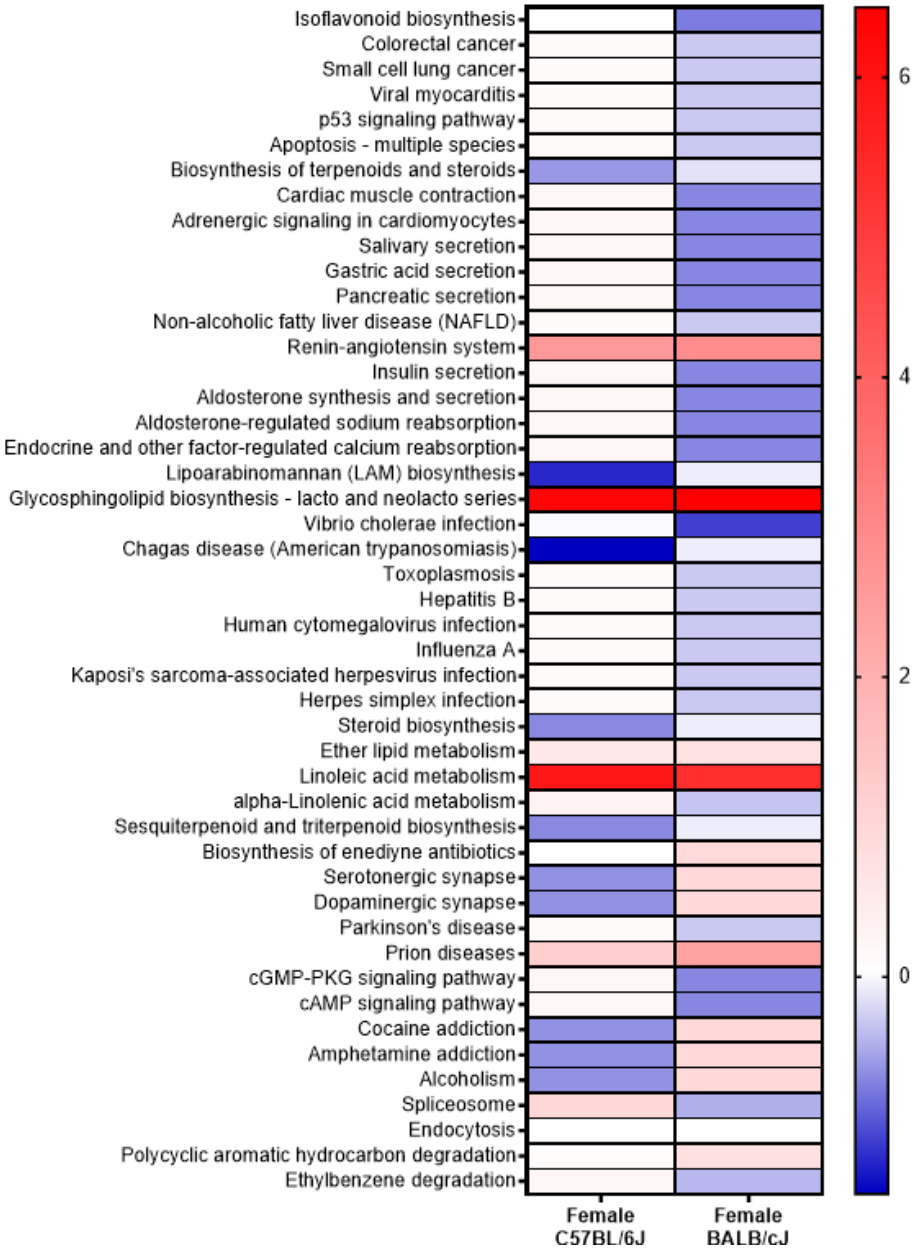
Supplemental Figure 6. Complete cytokine/chemokine array data using Quantibody Mouse Cytokine Array 5.



Supplemental Figure 7. Extended diversity indices. In addition to the observed species, Chao1, Shannon, and Simpson indices were used to compare the diversity between sex-matched groups.

Supplemental Figure 8. Complete KEGG Pathway analysis performed based on the sensitization-induced microbiome changes. Tax4Fun2 reference database was used to identify differential activation of pathways based upon changes in microbiome. Level 1 pathway classifications with an adjusted p-value < 0.05 (t-test with Bonferroni correction) and average $\log_2$ greater or less than 1 (fold changes greater or less than 2) are displayed in a heatmap grouped by level 2 classification.


Isoflavonoid biosynthesis
Colorectal cancer
Small cell lung cancer
Viral myocarditis
p53 signaling pathway
Apoptosis - multiple species
Biosynthesis of terpenoids and steroids
Cardiac muscle contraction
Adrenergic signaling in cardiomyocytes
Salivary secretion
Gastric acid secretion
Pancreatic secretion
Non-alcoholic fatty liver disease (NAFLD)
Renin-angiotensin system
Insulin secretion
Aldosterone synthesis and secretion
Aldosterone-regulated sodium reabsorption
Endocrine and other factor-regulated calcium reabsorption
Lipoarabinomannan (LAM) biosynthesis
Glycosphingolipid biosynthesis - lacto and neolacto series
Vibrio cholerae infection
Chagas disease (American trypanosomiasis)
Toxoplasmosis
Hepatitis B
Human cytomegalovirus infection
Influenza A
Kaposi's sarcoma-associated herpesvirus infection
Herpes simplex infection
Steroid biosynthesis
Ether lipid metabolism
Linoleic acid metabolism
alpha-Linolenic acid metabolism
Sesquiterpenoid and triterpenoid biosynthesis
Biosynthesis of enediyne antibiotics
Serotonergic synapse
Dopaminergic synapse
Parkinson's disease
Prion diseases
cGMP-PKG signaling pathway
cAMP signaling pathway
Cocaine addiction
Amphetamine addiction
Alcoholism
Spliceosome
Endocytosis
Polycyclic aromatic hydrocarbon degradation
Ethylbenzene degradation
Male C57BL/6J
Male BALB/cJ
1
0
-1
-2


Isoflavonoid biosynthesis
Colorectal cancer
Small cell lung cancer
Viral myocarditis
p53 signaling pathway
Apoptosis - multiple species
Biosynthesis of terpenoids and steroids
Cardiac muscle contraction
Adrenergic signaling in cardiomyocytes
Salivary secretion
Gastric acid secretion
Pancreatic secretion
Non-alcoholic fatty liver disease (NAFLD)
Renin-angiotensin system
Insulin secretion
Aldosterone synthesis and secretion
Aldosterone-regulated sodium reabsorption
Endocrine and other factor-regulated calcium reabsorption
Lipoarabinomannan (LAM) biosynthesis
Glycosphingolipid biosynthesis - lacto and neolacto series
Vibrio cholerae infection
Chagas disease (American trypanosomiasis)
Toxoplasmosis
Hepatitis B
Human cytomegalovirus infection
Influenza A
Kaposi's sarcoma-associated herpesvirus infection
Herpes simplex infection
Steroid biosynthesis
Ether lipid metabolism
Linoleic acid metabolism
alpha-Linolenic acid metabolism
Sesquiterpenoid and triterpenoid biosynthesis
Biosynthesis of enediyne antibiotics
Serotonergic synapse
Dopaminergic synapse
Parkinson's disease
Prion diseases
cGMP-PKG signaling pathway
cAMP signaling pathway
Cocaine addiction
Amphetamine addiction
Alcoholism
Spliceosome
Endocytosis
Polycyclic aromatic hydrocarbon degradation
Ethylbenzene degradation
Female C57BL/6J
Female BALB/cJ
6
4
2
0

Supplemental Figure 9. Complete microbiome profiles of all experimental groups. Found in Supplementary Data 6. of the following publication: https://pubmed.ncbi.nlm.nih.gov/33705867/

Supplemental Video 1.	Male Sham mouse carefully walking across the open zone of the EZM. Found in Video S1. of the following publication: https://www.frontiersin.org/articles/10.3389/fncel.2019.00320/full
Supplemental Video 2.	Male BLG mouse briefly surveying the open zone of the EZM and returning to the closed zone. Found in Video S2. of the following publication: https://www.frontiersin.org/articles/10.3389/fncel.2019.00320/full

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